Contemporary Oral Oncology

Biology, Epidemiology, Etiology, and Prevention

Moni Abraham Kuriakose
Editor

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To my parents for igniting the fire for gaining and sharing knowledge
To my teachers and colleagues for keeping that fire burning
To my students for keeping me on my toes
To Rohan and Mili for keeping me grounded
To my patients for enduring our quest for cure
To Maria for being a patient partner in this quest

MAK

Foreword

Writing and editing a comprehensive multivolume text and a reference source on a focused topic is a dream of a life time for scores of academicians, but only a handful are capable of and committed to realize that dream. Dr. Moni Abraham Kuriakose is to be commended to bring that dream to a reality in the field of oral cancer. He has successfully gathered an assembly of world-class leaders from all corners of the globe to contribute to this exhaustive four-volume treatise on the current state of the art and science of oral oncology. The organization and planning of such an in-depth reference source takes deep understanding of the biology of the disease, and mastery in clinical management of the patient. The editor in chief has very carefully selected scholars from the Roswell Park Memorial Institute, coupled with others from North America, Europe, and Australasia, in the specialty of oro-maxillo-facial surgery and oncology, to have a global perspective of the disease. This provides a global perspective from different geographic regions of the world, with diverse patient populations and varied socioeconomic and cultural differences.

Although, the commonly identified etiologic agents for oral cancer are prevalent throughout the world, the biological behavior and natural history of these tumors are different in various regions of the world. For example, the presentation and behavior of oral cancer seen in South Asia is quite different than that in the western world. The authors have very elegantly delved into the biology of these differences and have highlighted the frontiers in research in this area. Similarly, practical issues in the clinical management of patients in diverse socioeconomic regions are discussed to make this a valuable resource for clinicians throughout the world.

This four-volume, in-depth, and exhaustive text presents frontiers in current research in basic sciences and the biological basis of carcinogenesis, tumor progression, metastases, and recurrence. The breadth and depth of the biology of squamous carcinoma covered in the text by global experts is impressive. Equally well covered are the chapters on diagnosis, treatment, operative technical details, and outcomes: both functional and oncologic. Each chapter is well illustrated with photographs, and superb artwork, to convey to the reader the intricate details from biological processes, to surgical techniques. Each and every chapter is accompanied by an endless list of references, to make this a "go to" resource and a reference text on the topic. This opus of oral oncology from molecular signatures to CAD-CAM technology in reconstructive surgery is a one of a kind publication on this subject published in a long time.

The four-volume set in *Contemporary Oral Oncology*, will have a solid place in the libraries of medical schools, postgraduate institutions, Cancer centers, and specialty departments in Universities. It is a wonderful state-of-the-art resource for the trainee as well as the practitioners of oral oncology, to remain current with the topic, and as a ready reference in basic and clinical research as well as day today management of patients. This exhaustive work stands alone in the presentation of biology, diagnosis, clinical care, prevention, and outcomes in oral cancer.

New York, NY, USA

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Preface

Oral oncology is emerging as a distinct discipline. Comprehensive management of oral cancer requires multidisciplinary input of interconnected specialties. Every aspect of the management from diagnosis, treatment, reconstruction, and rehabilitation has biological basis. The biologic understanding of oral cancer and the treatment is changing with time. Understanding and updating developments in each of the related fields are essential to offer the patients the best possible treatment.

This book, in four volumes, is an in-depth reference guide that covers all aspects of the management of oral cancer from a multidisciplinary perspective and on the basis of a strong scientific foundation. Individual volumes are devoted to tumor biology, epidemiology, etiology, and prevention; diagnosis and treatment options; reconstructive surgical techniques; and rehabilitation and supportive care. By integrating current scientific knowledge into a manual for comprehensive care of the oral cavity cancer patient, this book is expected to fill a substantial void in the literature. Further key features are attention to the practical significance of emerging technology and the inclusion of contributions from authors in diverse geographic locations and practice settings in order to ensure that the guidance is of global relevance. The text is supported by ample illustrations and by case studies highlighting important practical issues.

There is lack of a single multidisciplinary comprehensive reference guide in oral oncology. This book is envisioned to fill this substantial void in literature. This book is intended for both trainees and practicing specialists in oral oncology. During my training, clinical practice, and research, I had the opportunity to gain knowledge and skills from different disciplines that includes dentistry, medicine, oral and maxillofacial surgery, general surgery, otolaryngology, plastic surgery, and basic science research spanning three continents. This unique opportunity provided me an insight into the importance of cross-fertilization of ideas from different disciplines and geographic regions. This book is an attempt to impart that principle to the field of oral oncology.

The first volume is dedicated to tumor biology, epidemiology, etiology, emerging role of cancer stem cells, and the prevention of oral cancer. It opens by discussing oral carcinogenesis in general and the role of different carcinogens and human papillomavirus in particular. Global epidemiology and changes in disease prevalence are then addressed. Up-to-date information is provided on emerging cancer

biomarkers, and the biologic basis of personalized therapy is explained. Histopathological features of malignant and premalignant neoplasms and their relevance to management are described. Further chapters focus on the current status of chemoprevention, the management of oral submucous fibrosis, and the value of various diagnostic adjuncts. This volume concludes by critically evaluating the efficacy of oral screening methods.

The second volume deals with diagnosis and management of oral cancer. This volume addresses a range of management issues in oral cancer, from imaging and staging through to the roles of radiation therapy and chemotherapy. Principles of ablative surgery are explained, and neck dissection and sentinel lymph node biopsy techniques are described. Detailed consideration is also given to the management of complications, salvage surgery and re-radiation, the biologic basis of treatment failure, and emerging approaches to overcome treatment resistance. The inclusion of resource-stratified guidelines will meet the needs of practitioners in different geographic regions with varying resources.

The third volume is devoted to the reconstructive surgical techniques used in patients with oral cancer. Following introductory chapters outlining the general principles of reconstructive surgery in the oral cavity and the planning of maxillofacial reconstruction, detailed descriptions of the options and techniques employed in reconstruction of each of the functional subunits are provided. Important technologic advances are also discussed, including image-guided surgery, robotic surgery, and tissue-engineered and prefabricated approaches. Finally, the current status of face transplantation for maxillofacial reconstruction is reviewed.

The last of this four-volume book deals with the most important and often neglected aspect of rehabilitation and supportive care. This volume focuses on the topic of comprehensive rehabilitation and supportive care in oral cancer. The coverage includes the role of maxillofacial prosthodontics, advances in anaplastology techniques, and management of oral mucositis during radiation and chemotherapy. Holistic and supportive care approaches are discussed, and advice is provided on post-therapy surveillance and the use of different measures to assess quality of life. Nutritional evaluation and management and issues relating to healthcare economics are also considered. This volume will be of interest both to practicing specialists and to ancillary service staff involved in the care of oral cancer patients.

This book was authored by leaders in the field from diverse medical disciples and geographic regions. I thank the authors whose expertise and hard work that has distilled a vast body of information into a clear and detailed discussion of various aspects of oral oncology. I would like to express my thanks to the Springer Nature for supporting me in developing this book, to Wilma McHugh for project management and constant support, and to Abha Krishnan and Eswaran Kayalvizhi for the editorial assistance.

I have personally benefitted immensely by the tutelage of many mentors notably Sripathy Rao, Paul Salins, K. Kamalamma, Adrian Sugar, Anwar Perriman, Montague Barker, Paddy Smith, Brian Awry, John Hawksford, Keith Postlethwaite, Leo Stassen, Ian Martin, Andrew Ryan, Collin Edge, Mark DeLacure, Wesley Hicks Jr., Thom Loree, Richard Bankert, and my colleagues at New York University:

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Buffalo, NY, USA

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Contents

Carcinogenesis and Field Cancerization in Oral Squamous Cell Carcinoma

1

Amritha Suresh, Moni Abraham Kuriakose, Simple Mohanta, and Gangotri Siddappa

1.1 Oral Carcinogenesis

Oral carcinogenesis is a multistep, multifocal process initiated as a consequence of carcinogenic insults on the oral mucosa in individuals with genetic susceptibility for oral cancer. The carcinogenic process results in successive molecular changes that lead to dysregulation of cell proliferation, growth, and differentiation. The changes at the genetic and molecular levels ultimately lead to cellular transformation and carcinogenesis. The carcinogenic process in oral cancer, as is the case with majority of other solid tumors, occurs stepwise fashion at molecular, histological, and clinical levels.

Clinically a significant proportion of oral cancers develop as white patch (leukoplakia), mixed white and red patch (speckled leukoplakia), and red patch (erythroplakia). There is higher rate of dysplastic lesions in erythroplakia as compared to leukoplakia. A subset of leukoplakia with verrucous surface morphology called proliferative verrucous leukoplakia has the highest malignant transformation potential. A significant number of oral squamous cell carcinomas develop in clinically normal mucosa. In this scenario it is assumed that the molecular changes of oral carcinogenesis has not lead to changes in the appearance of the oral mucosa. Novel diagnostic adjuncts are being developed to detect these sub-clinical lesions. Therefore clinical appearance does

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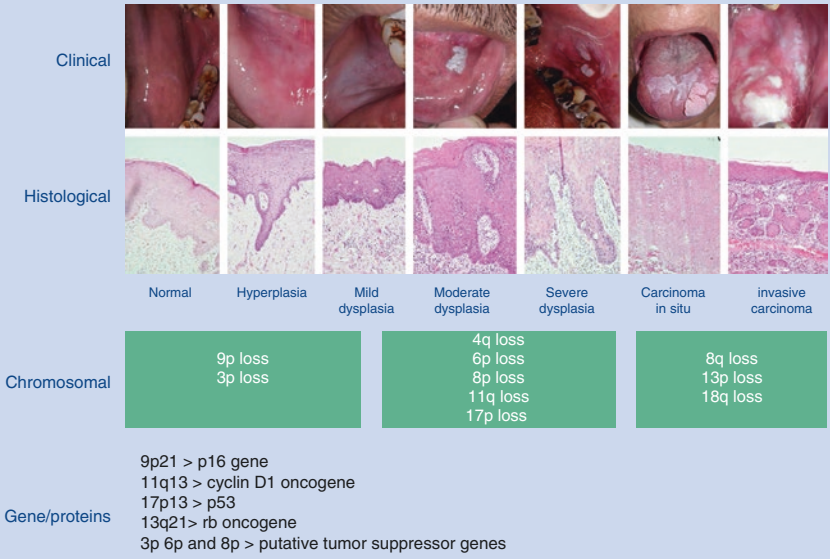
not correlates with histology of lesions. A biopsy is essential to make management decision. At the histological level, the disease progresses from epithelial hyperplasia and hyperkeratosis, mild dysplasia, moderate dysplasia, severe dysplasia to carcinoma in situ and invasive carcinoma. It is to be noted that not all the lesions progress linearly on one direction as many dysplastic lesions can reverse to non-dysplastic lesions. On a chromosomal level, some of the early changes are seen at loss of 9p and 3p loci. With dysplastic lesions, loss of 4q, 6p, 8p, 11q, and 17p loci are seen. In invasive carcinoma, 8q, 13p, and 18q loss are seen. Corresponding changes in genes are also observed during malignant transformation. These include p16, cyclin D1, p53, and pRb



1.1.1 Clinical Progression of Oral Cancer

The concept of a two-step carcinogenesis process in the oral mucosa is well established [1]. The initial dysplastic changes lead to the development of premalignant lesions which then develop into carcinoma. The potentially malignant lesions have varying rates of malignant transformation potential, adding to the challenge of an accurate detection of susceptible lesions. However, evidences also exist that point out to the development of oral carcinoma without being preceded by clinically overt premalignant lesions.

1.1.1.1 Potentially Malignant Lesions

The two well-known types of oral premalignant lesions (PMLs) with varying rates of transformation are leukoplakia (2–8 %) and erythroplakia (14–67 %) [2]

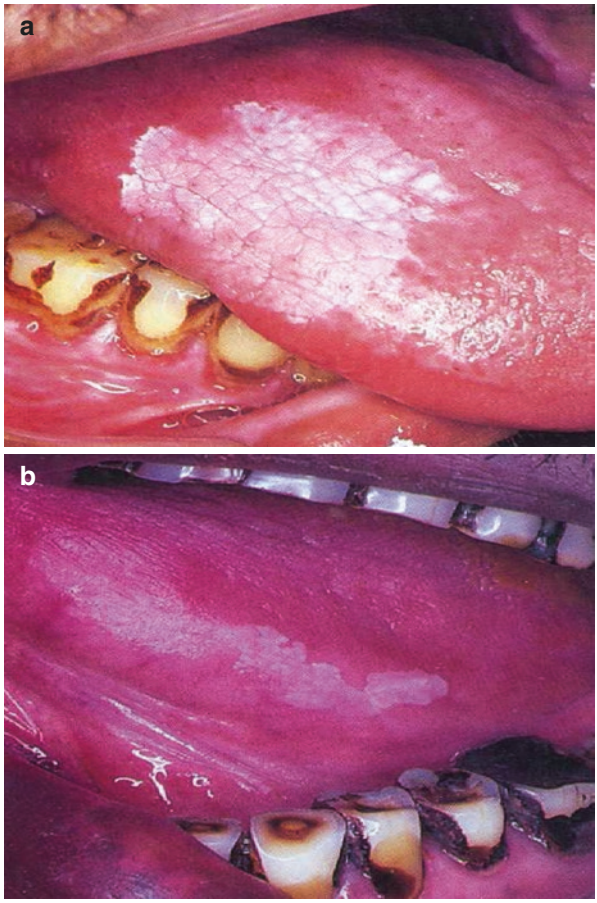


Fig. 1.1 Clinical appearance of leukoplakia (**a**) and erythroplakia (**b**)

(Fig. 1.1a, b). Leukoplakia can be defined as "white patch or plaque" that cannot be characterized clinically or pathologically as any other disease and is not associated with any physical or chemical causative agent except the use of tobacco [3], whereas erythroplakia is a clinical term given for a chronic red mucosal macule that looks similar to leukoplakia, but which cannot be attributed to traumatic, vascular, or inflammatory causes [3, 4]. Nevertheless, erythroplakia is less common than the white precancerous lesions, and on careful observation, they are found to be associated with many early invasive oral carcinomas. A variant of oral leukoplakia was recently described called proliferative verrucous leukoplakia (PVL) with very high prevalence of malignant transformation. PVL can be defined as a progression of white mucosal plaques that virtually always develops into nodular, papillary, or verruciform surface projections and gradually, sometimes rapidly, spreads laterally to cover up large regions of oral mucosa. It was also reported that PVLs have high transformation rate (>70 %) when compared to other potentially malignant lesions [5, 6].

Other types of potentially malignant conditions include oral submucous fibrosis (OSMF) and lichen planus. It was observed that OSMF is strongly associated with the chewing habit (areca nut, betel quid, gutka) and is an irreversible precancerous lesion with a transformation rate of 5 % [7]. It was shown in a study that patients with both oral leukoplakia and oral submucous fibrosis (OSMF) are at higher risk for malignant transformation into oral cancer when compared to the presence of either of these lesions alone, the risk for leukoplakia being the most significant [8]. Lichen planus, an autoimmune disorder of the oral mucosa and skin, typically represented as intertwining, thin strands or streaks of white keratosis (Wickham's striae) with a very low rate of transformation (<1 %) [9, 10].

1.1.2 Histological Progression

The histological neoplastic progression is based on the grade of dysplasia. This is determined by multiple tissue architecture (epithelial stratification, polarity, mitotic figures, keratinization, keratin pearls) and cellular criteria (nuclear/cytoplasmic ratio, nuclear pleomorphism, and size). The hierarchical gradation of histological progression is from normal to hyperplasia, to mild, moderate, and severe dysplasia. This is subsequently followed by carcinoma in situ which ultimately progresses into squamous cell carcinoma.

The major cellular-based changes during progression from normal to hyperplasia, dysplasia, carcinoma in situ, and squamous cell carcinoma include abnormal variation in nuclear size and shape (anisonucleosis and pleomorphism), increased nuclear/cytoplasmic ratio, enlarged nuclei and cells, hyperchromatic nuclei, increased mitotic figures, abnormal mitotic figures (abnormal in shape or location), and increased number and size of nucleoli [11]. These changes evident in the epithelial cells can enable the identification of atypical cells during the process of carcinogenic progression (Table 1.1) [12].

The variability of these changes depends on the grade of dysplasia. Similarly, there are few major architectural/tissue-based changes that can be observed during the progression which include loss of polarity, disordered maturation from basal to squamous cells, increased cellular density, basal cell hyperplasia, dyskeratosis (premature keratinization and keratin pearls deep in the epithelium), bulbous drop-shaped rete pegs, secondary extensions (nodules) on rete tips, and top-to-bottom change of carcinoma in situ [11]. These tissue-based and cellular-based changes involved in each grade of dysplasia are as mentioned below in Table 1.2.

Table 1.1 Groups showing light microscopic and scanning electron microscopic difference in epithelium and epithelial cells [20]

Groups studied	Light microscopic study (difference in epithelium)	Scanning electron microscopic study (difference in epithelial cells)
Normal mucosa	Non-keratinized stratified squamous epithelium	Flat-surfaced cells with equidistant parallel micro ridges
Oral mucosa exposed to tobacco/alcohol	Hyper-para-keratinized stratified squamous epithelium with mild cytological atypia	Irregular and widened micro ridges with numerous pits and absence of honeycomb pattern
Clinically diagnosed leukoplakia	Architectural and cytological changes	Irregularly arranged broad and swollen cells with numerous pits and irregular microvilli projecting over the surface

Table 1.2 Cytological and architectural changes during progression of the disease from normal to hyperplasia, dysplasia, and carcinoma in situ [21]

Grade	Levels involved	Cytological changes	Architectural changes
Hyperplasia	N/A	None	Thickened epithelium Hyperkeratosis Normal maturation Basal cell hyperplasia
Mild	Lower third	Cellular and nuclear pleomorphism Nuclear hyperchromatism	Basal cell hyperplasia
Moderate	Up to middle third	Cell and nuclear pleomorphism Anisocytosis and anisonucleosis Nuclear hyperchromatism Increased and abnormal mitotic figures	Loss of polarity Disordered maturation from basal to squamous cells Increased cellular density Basal cell hyperplasia Bulbous drop-shaped rete pegs
Severe	Up to upper third	Cell and nuclear pleomorphism Anisocytosis and anisonucleosis Nuclear hyperchromatism Increased and abnormal mitotic figures Enlarged nuclei and cells Hyperchromatic nuclei Increased number and size of nucleoli Apoptotic bodies	Disordered maturation from basal to squamous cells Increased cellular density Basal cell hyperplasia Dyskeratosis (premature keratinization and keratin pearls deep in epithelium) Bulbous drop-shaped rete pegs Secondary extensions (nodules) on rete tips Acantholysis
Carcinoma in situ	Full thickness	All changes may be present	Top-to-bottom change Loss of stratification

1.1.3 Molecular Model of Progression

Knudson two-hit hypothesis: Chromosomes are represented in pairs in the normal cells, one inherited from the mother and the other from the father. All the genes have representation on both the chromosomes of a pair in the form of alleles. The first hit of carcinogenic insult can cause loss or mutation of an allele in one of the chromosomes. The first hit is usually thought of as a point mutation that inactivates one copy of a tumor suppressor gene, such as Rb1. The individual does not develop cancer at this point because the remaining tumor suppressor gene allele on the other locus is still functioning normally. This loss of heterozygosity is common in many cancers. Continued carcinogenic insult can lead to loss of the remaining normal gene (second hit) leading to loss of function of that gene. If this happens to be, a tumor suppressor gene (e.g., p53), it can affect DNA repair mechanism and the integrity of the entire genome leading to development of cancer.

In hereditary cancers (e.g., xeroderma pigmentosa where nucleotide excision repair enzyme gene is defective), the individual is born with one mutated gene. Carcinogenic insult can cause deletion of the normal functioning gene leading to the development of cancer. This two-step process of genetic basis for carcinogenesis is called Knudson two-hit hypothesis.

The early molecular models developed based on marker correlation with stepwise histological progression changes [12–14] indicated that specific molecular changes associated with each stage of histology. Loss of heterozygosity (LOH) [see definition] one of the earliest chromosomal abnormalities to be associated with cancer at specific sites correlated with histological progression. LOH at 3p and 9p along with allelic instability at both the loci is one of the biomarkers identified as the initial steps of malignant transformation [15–19]. LOH at 9p with TP53 mutation was also shown to be associated with malignant transformation and can be similarly used as a biomarker for malignant transformation prediction [20]. Recent studies have also reported that LOH at 15 microsatellite markers [3p, 9p, 17p, 8p, 13q and 18q] was observed frequently in histologically higher-grade lesions (moderate or severe dysplasia) and in lower-grade lesions (mild dysplasia) when there is a high proliferation rate [21]. It was also shown that LOH at 4q, 8p, 11q, and 13q was significantly associated with presentation of dysplastic lesions along with marginal significance for LOH at 17p, whereas LOH at 4q, 8p and 17q were associated with hyperplasia, 11q LOH showing a marginally signficant association [18]. Studies over the past decade have also identified various molecular players associated in the initial neoplastic development and subsequent progression to carcinoma.

In addition to these changes that were documented as essential during the stepwise progression of oral carcinogenesis, studies have identified many molecular players that contribute toward the initiation and progression of oral cancer.

1.1.4 Biomarkers of Oral Carcinogenesis

1.1.4.1 Tumor Suppressor Genes (TSG)

Deregulation of the tumor suppressor gene family is one of the earliest events in initiation of tumorigenesis. Molecular changes in p53/Rb and the p16/pRb/cyclin D1 pathway are known to lead to acquisition of dysplastic characteristics [22]. Correlation with patients at various stages of oral cancer progression has been the primary mode of understanding the role of markers during the process. Multiple studies have shown that the expression of suprabasal p53 is associated with different grades of dysplasia [23–25]. CDK1 (p21) is another TSG that correlated with early dysplastic progression with the gene showing a significant and progressive increase in expression from mild (3 %) to moderate (50 %) and severe dysplasia (64 %) [26]. P27 on the other hand showed a positive suprabasal staining pattern in normal and mild dysplasia that became less apparent with increasing degrees of dysplasia [26–28]. Among the Rb family of TSGs, a significant loss of Rb and p16 levels was reported at the transition from hyperplasia to dysplasia [22, 26, 29, 30].

Progression of dysplastic lesions to carcinoma mostly involves a further increase in cell proliferation accompanied by an increase in properties of migration and invasion, ultimately leading to metastasis. Multivariate analyses in different studies has shown that although deregulation of p16/pRb/Cyclin D1 pathway is an early event in dysplasia development, both pRb and p53 pathways are associated with malignant transformation and adverse prognosis in oral cancer [22, 31]. Genetic alterations identified in the retinoblastoma (Rb) family members, pRb, pRb2/p130, and p107, are reported to be involved in growth arrest, apoptosis, differentiation, and angiogenesis that may act as significant factors for pathogenesis and progression of tumor in various cancers and, hence, may be useful for assessing the risk in cancer patients. Expression of pRb2/p130 may be inversely correlated with malignancy of oral dysplastic lesions and, hence, can be used as an indicator for progression [32, 33]. PTEN, a candidate tumor suppressor gene located at 10q23.3, might also play an important role since lack of PTEN expression is an independent prognostic indicator for clinical outcome, as observed in patients with tongue cancer [34].

1.1.4.2 Cell Cycle and Proliferation Markers

Cell cycle deregulation is one of the major means by which malignant transformation is affected; the pathways and molecules involved in this process hence show differential regulation during the various stages of oral carcinogenesis. It was shown that a combination of elevated expression of cyclin-dependent kinases (cyclin D1, cyclin E, CDK2) and loss of epigenetic markers [p12 (DOC-1), p16 (INK4A), p27

(KIP1)] may contribute to the multistep nature of oral carcinogenesis [26]. p27Kip1, a member of the CIP/KIP family of CDK inhibitors that negatively regulates cyclin-cdk complexes, was found to show reduced expression and was associated with increased cell proliferation, although other changes might contribute to altered cell kinetics during carcinogenesis [27].

Minichromosome maintenance protein (MCM2-7) is essential for eukaryotic replication initiation and along with another cell cycle protein, Geminin, are suggested to be novel biomarkers of growth and proliferation in oral epithelial dysplasia [35, 36]. Other cell cycle proteins such Cdc6 were also overexpressed, with the expression correlating to the development and metastasis of oral cancer suggesting that it can be a molecular marker for early diagnosis and prognosis prediction [36].

Suprabasal expression of Ki-67, an indicator of proliferation, was directly associated with the presence and severity of oral dysplasia [37, 38]. It was reported that oral dysplasia is characterized by lower cell proliferation and a higher frequency of cell death when compared to SCC, and moreover, several indices combining the expression of multiple markers are known to be indicators of dysplastic development. High labeling indices (LI) of minichromosome maintenance 2 (MCM2) and p53 and lower LI of p21 are suggested to be helpful in the prediction of malignant transformation of oral dysplasia and also as a biomarker of proliferating cells [39].

1.1.4.3 Angiogenesis and Metastatic Markers

Angiogenesis is vital to the malignant transformation process; molecules that facilitate the process are thus possible indicators of oral cancer progression. Studies in premalignant lesions have reported Willebrand factor along with p53 to be associated with oral carcinogenesis [40]. Microvascular density (MVD), an indirect marker of neo-angiogenesis, as detected by markers CD31, CD34, and CD105, is known to be significantly associated with different grades of dysplasia [41–43]. Studies have also associated the markers of angiogenesis such as VEGF with the late progression of oral cancer, recurrence, and metastasis rather than the early stages [44, 45].

Tumor protein 63, a p53 homolog, is highly expressed in the nuclei of basal regenerative cells and was commonly upregulated in HNSCC and subsequently resulting in the increased expression of downstream molecules such as MMP14 and LAGLS1, motility-related molecules indicating its efficacy to determine potential metastatic tumors [46, 47]. The increase in p63 and CD105 expression has also been correlated with a concomitant loss of membranous E-cadherin indicating an association with increased EMT behavior [48]. Ezrin, a member of the ERM protein family, plays key roles in cell structure, organization, adhesion, and migration. The Akt/Ezrin Tyr353/NF-κB is known to regulate EGF-induced EMT and metastasis in tongue cancer; EZRIN is suggested to be a therapeutic target to reverse EMT and prevent progression [49].

1.1.4.4 Cytokeratins

Cytokeratins, essential for the maintenance of the cytoskeletal assembly, are found to be extensively overexpressed in HNSCC [50]. The expression pattern of cytokeratin filaments in the epithelium was found to be directly dependent on the type

and differentiation pattern of tumors [51]. CK-10/CK-11 and involucrin that are normally present in terminally differentiating keratinocytes showed strong correlation with the differentiation status of cells: high expression in non-dysplastic hyperplastic epithelium as compared to normal, dysplastic, and neoplastic epithelium. These proteins were also found to be inversely correlated with various grades of dysplasia suggesting that these proteins may be useful biomarkers for epithelial carcinogenesis [52].

1.1.4.5 miRNA Markers

miRNAs are now increasingly implicated in various aspects of carcinogenesis; studies in oral cancer have also revealed their role in malignant transformation. miR-21, miR-181b, and miR-345 were found to be consistently increasing in expression with the increase in severity of the premalignant lesion. Upregulation of miR-181 in OSCC during its progression from leukoplakia to dysplasia to invasive carcinoma was correlated with lymph node metastasis, vascular invasion, and poor survival since upregulation might enhance migration [53, 54]. miRNA markers that are differentially expressed in tissue and saliva with concordant fold levels can be used for monitoring of potential relapse or malignant transformation in oral cancers [49, 55–57].

hTERT, the human telomerase reverse transcriptase, a component of the Telomerase complex, is known to have an elevated expression profile in oral cancers as compared to the normal oral mucosa. Other studies have also shown that this increased expression of hTERT protein was found to be an early event in oral carcinogenesis, and the amount of cytoplasmic or nuclear expression of hTERT was an accurate indicator of progression, recurrence, and prognosis in OSCC [58–62].

Studies have shown that osteopontin (OPN), a secreted, chemokine like protein, can be used as a prognostic marker for OSCC and not for progression since the expression in PMLs was not in accordance with their histological grading and the intensity of expression was also similar to that seen in normal epithelium [63]. Expression of Nuclear factor KB (NF-KB) and Cyclooxygenase 2 (COX-2) proteins, known to be regulated by Osteopontin were found to increase with histological progression of the disease (normal to leukoplakia to carcinoma). It is also reported that NFKB shows a negative correlation in tumor-surgical margin-to extra margin, with COX-2 showing a parallel expression. These studies suggest that NFKB might be involved in the later stages of acquisition of malignant phenotype in oral carcinogenesis while COX-2 may be involved at the early stages [64, 65].

EGFR is a cell surface receptor to which ligands such as epithelial growth factor (EGF) bind. Once activated, it undergoes a fully reversible dimerization to form a homodimer [66]. Deregulated mutant EGFR overexpression was observed in majority of patients with HNSCC and is reported to enhance tumorigenic capabilities [67]. An increased copy number in EGFR gene can be used for the prediction of

malignant transformation in oral premalignant lesions [68]. In oral premalignant lesions, with the expression being higher in high risk lesions.

Claudins, normally expressed in a reticular pattern up to the prickle layer in normal mucosal epithelium, are directly correlated with the grade of tumors and vascular infiltration and inversely correlate with recurrence; Claudin 7, one of the member of the family reported to be a poor prognosticator in Oral cancer [69]. Melanoma-associated antigen-A (MAGE-A), an antigen restricted to malignant cells, can be used as a marker in high-risk patients for an accurate estimation of potential malignant transformation of premalignant lesions [70]. The advanced oxidation protein products (AOPP) obtained from different oxidation patterns were known to produce of either NO or H_2O_2 which leads to the generation of different types of reactive oxygen species that set a cascade of reactions with a potential to damage cellular micromolecules eventually turning out into frank OSCC [71]. Some of the other markers that are known to be associated with early dysplastic progression are the WW-domain-containing oxidoreductase (WWOX) with >35 % of the dysplastic lesions showing altered transcript and protein levels. A combined expression of stromelysin and Ets-1 was shown to be predictive of transition to a precancerous stage with high statistical significance [72].

Oncoprotein Bcl-2 regulates programmed cell death by allowing tumor cells to escape apoptosis and was found to be overexpressed in OSCC as compared to premalignant lesions suggesting its presence in the early stages of carcinogenesis [73]. It was shown that Bax and Bcl-X along with p53 were expressed early, and Bcl-2 and MDM-2 showed sporadic expression in the development of oral premalignant and malignant disease suggesting that protein regulation of apoptosis may be altered during the development of OSCC [23]. An inverse relationship was found between Bcl-2/Bax ratio and apoptosis from normal oral epithelium to severe dysplasia indicating that suppression of Bcl-2 may have a role in oral tumorigenesis [74].

Podoplanin, a mucin-like transmembrane glycoprotein specifically overexpressed in lymphatic endothelial cells, was found to be expressed in hyperplastic and dysplastic areas adjacent to primary tumors indicating that its abnormal expression occurs early in oral tumorigenesis [75]. The subcellular localization of the nuclear S100A7 gene, the calcium binding protein, was found to be expressed in early stages of oral premalignant lesions and was known to be a potential determinant for transformation of oral premalignant lesions and recurrence in HNSCC [76].

1.1.5 Cancer Stem Cells in Oral Carcinogenesis

At normal physiologic condition, stem cells localized at the basal layers are in a tightly regulated quiescent state. These cells undergoes asymmetric cell division with one daughter cell remaining as stem cell and the other as differentiated cells that maintain the epithelial integrity. After epithelial injury, stem cells lose its

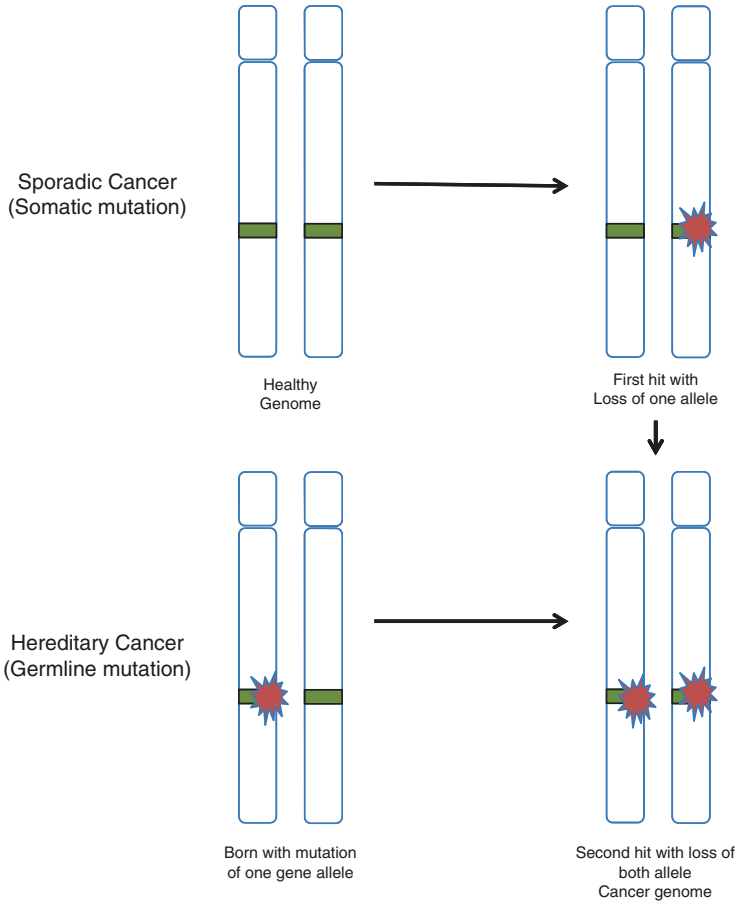


Fig. 1.2 Diagrammatic representation of Knudson two-hit hypothesis

inhibitory signal transiently and produce differentiated cells to repair the wound (Fig. 1.2). Since stem cells are the only long-term resident cells in an epithelium, they are likely to be the target of carcinogenic stimuli. By acquiring series of genetic and epigenetic changes, these stem cells transform into cancer stem cells (CSCs). Another view on origin of CSC is that they develop by dedifferentiation of tumorigenic epithelial cells (Fig. 1.3). In addition to the properties of normal stem cells, it acquires several other characteristics that make them resistant to inhibitory growth signals (Fig. 1.2). These include (a) self-sufficient growth signaling, (b) antigrowth signaling insensitivity, (c) evasion of apoptosis, (d) unlimited replication potential, (e) sustained angiogenesis, (f) and tissue invasion and metastasis [77–79].

Like normal stem cells, CSC are suggested to reside in a specific niche which is constituted mostly by the endothelial cells and the fibroblasts. The CSC-niche cross talk, though not extensively investigated in head and neck cancer, is suggested to be

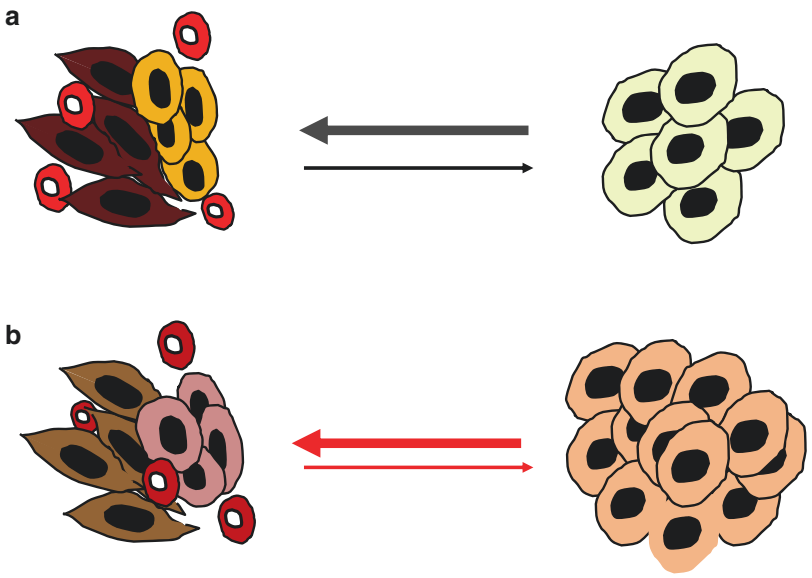


Fig. 1.3 (**a**) Stem cell niche is required for the maintenance of normal epithelial integrity, where the stem cells that are normally in quiescent stage transiently get activated to produce differentiated cells. (**b**) Cancer stem cells on the other hand have lost its negative feedback and are always on a active state

orchestrated by multiple pathways such as the TGFB1 signaling, SDF-1/CXCR4 axis and NPTCH1 signaling. Evidence does suggest that this cross-talk can induce CSC-like properties in the cancerous epithelial cells and/or initiate the development of cancer-associated fibroblasts/endothelial cells through secreted cytokines (eg. CXCL12, TGFB1) and their receptors (eg. CXCR4). The niche can hence play a major role in the carcinogenic process (Figs. 1.3, 1.4 and 1.5).

The cancer stem cell concept in oral carcinogenesis has been supported by the identification of markers that are associated with the early stages of oral cancer development. OCT-4, a protein encoded by the POU5F1 gene, was associated with worse survival rates, and low expression leads to loss of pluripotency [80, 81]. It was also found that ectopic expression of OCT-4 leads to dysplasia in adult mice tissues [82, 83]. Oral premalignant cells also show upregulation of EMMPRIN (CD147) when compared to normal oral epithelial cells. The expression correlates with the degree of dysplasia suggesting that overexpression of EMMPRIN occurs at a very early stage of oral carcinogenesis [84].

Aldehyde dehydrogenase 1 (ALDH 1), an intracellular enzyme, has been a cancer stem cell marker with its expression being higher in OSCC than normal mucosa [2, 85, 86]. ALDH1 and CD133 were also shown to serve as predictors in the identification of oral leukoplakia susceptible to development of oral cancer [87]. Expression of Nanog and Nestin and concurrent levels of OCT4 and SOX-2 were

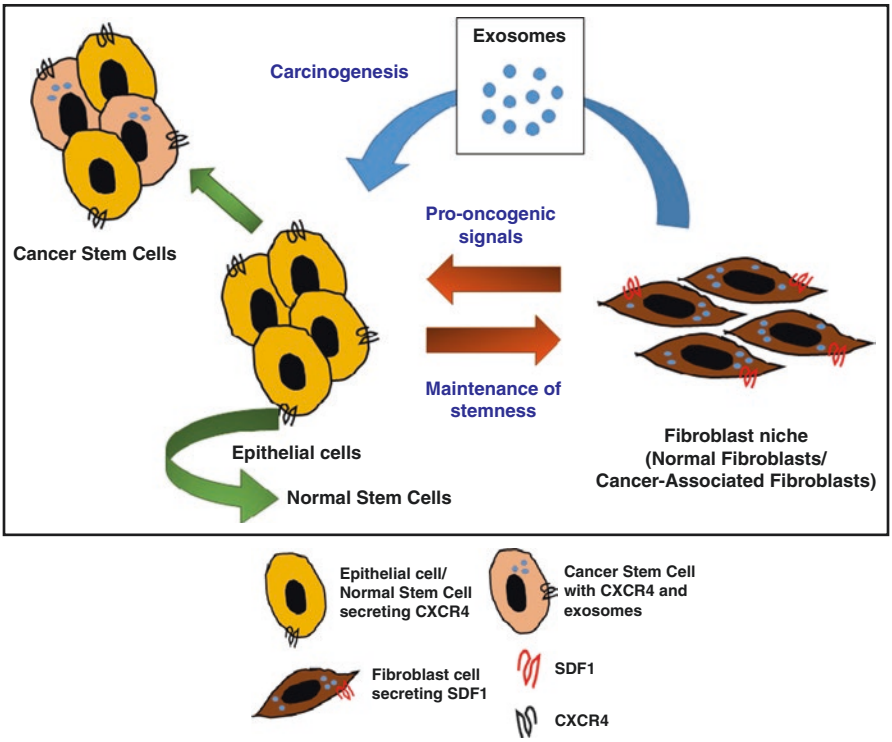


Fig. 1.4 In normal physiologic state, the epithelial stem cells homeostasis is maintained with its interaction with fibroblasts and endothelial cells. In cancer, the fibroblasts develop special features to acquire the phenotype of cancer-associated fibroblasts. The cross talk between cancer stem cells and cancer-associated fibroblasts is explained with the SDF-1/CXCR4 pathway as an example; CXCR4 being expressed on the Cancer stem cells and the SDF-1 being secreted by the niche

associated with low survival rates, aggressive growth, metastasis, and poor prognosis [88, 89]. CD44, a marker for OSCC stem cells, is known to be capable of inducing metastatic properties in nonmetastatic tumor cells [78, 90, 91]. Studies have also shown a gradual increase in the expression of the stem cells markers; CD133 and Musashi-1 observed from normal to dysplasia to carcinoma as well as in advanced and poorly differentiated tumors suggest the involvement of these proteins in oral carcinogenesis [92].

1.1.6 Markers of Oral Carcinogenesis: Implications in Early Detection and Chemoprevention

Early diagnosis is one of the major strategies that can help toward downstaging the disease at presentation and thereby improving survival rates in oral cancer. Advances in the molecular understanding of oral carcinogenesis thus can lead to

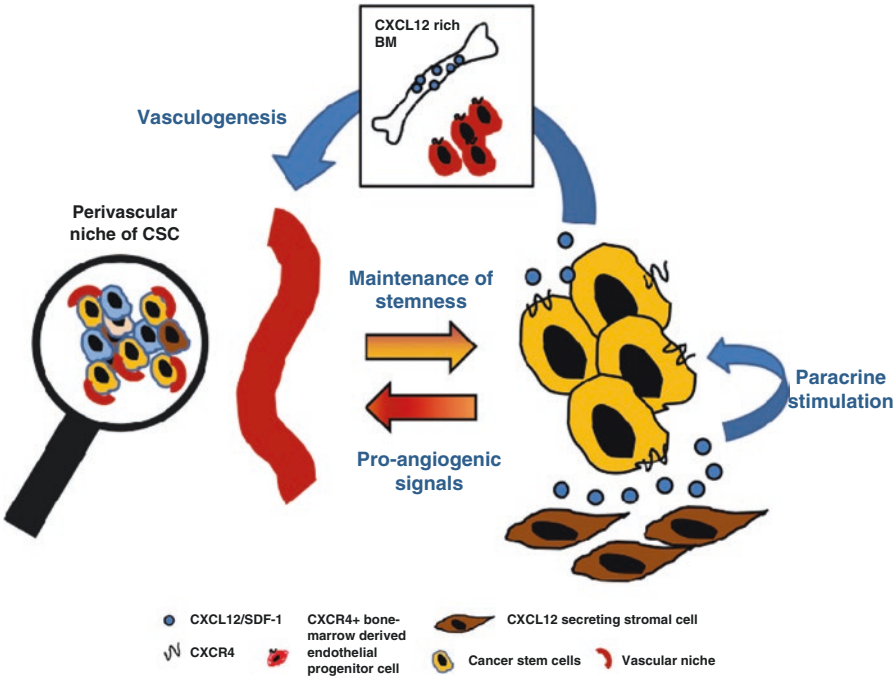


Fig. 1.5 The possible components and interaction of cancer stem cell niche

the identification of potential biomarkers that can be possible candidates for diagnosis. Nevertheless, the markers that have been identified have not been validated for use as diagnosis markers with confirmation by biopsy and subsequent histology being the gold standard. Lectin-based molecular imaging methods utilizing differences in glycosylation have indicated their utility as a diagnostic method [93]. Ongoing studies have also identified markers in saliva and serum that can be assessed for their utility for early detection [94–96]. However, extensive prospective validation studies are mandatory if these markers are to be applied in clinical practice.

Markers of oral carcinogenesis also pave the way to assess novel targets for chemoprevention, the primary strategy that can work toward improving the survival rates of the disease. Several molecules have been tested for their anti-tumorigenic activity in oral cancer. Studies using retinoids, known to differentiate cancer stem cells, showed an average 50 % response in patients with oral leukoplakia [97, 98]. Curcumin that acts on the NFkB pathway is also known to have chemopreventive characteristics as observed in studies on multiple solid tumors including oral cancer [99–101]. Nonetheless, studies are warranted to evaluate the efficacy of targeting the known markers of oral carcinogenesis toward chemoprevention and to also identify other potential novel targets.

1.2 Field Cancerization in Oral Cancer

Incidence of second primary tumors in oral cancer (10–30 % of the cases) occurring at the primary site, despite a complete resection of primary tumor, remains one of the key and challenging issues associated with oral cancer pathogenesis. Clinical studies indicate that "transformed cells" with the ability to initiate new tumors do exist in a histologically normal field surrounding the primary tumor. "Field cancerization," a term coined by Slaughter in 1953, proposes that adjacent normal tissue of tumor harbor certain preneoplastic genetic fingerprints which can eventually lead to local recurrence or second primary tumors, depending on the duration within which the tumor develops. Slaughter and his group based the concept on the following evidences: (1) oral cancer develops in multifocal areas of precancerous changes due to a prolonged and widespread exposure to carcinogens, (2) "abnormal" tissue surrounds the tumor, (3) oral cancer often consists of multiple independent lesions that sometimes coalesce, and (4) the persistence of abnormal tissue after surgery may explain the formation of second primary tumors and local recurrences [102].

It is well known that the onset of carcinogenesis begins long before the clinical detection of the cancerous lesions in the tissue. The detection of morphological changes of cancerous origin occurs at a much later stage during carcinogenesis. Adjacent mucosa surrounding the tumor, though histologically normal, has been shown to have precancerous changes, and these modifications have been suggested to be the cause for the formation of second primary tumors and local recurrence [103, 104], which subsequently lead to poor survival and an increase in mortality rate. Histologically normal cells thus can also harbor the tools and means for cancer formation; most of the studies have proven field cancerization to be one of the reasons behind the recurrence of the disease in the primary as well as at secondary locations. The concept drives the notion of precancerous cells replacing the normal epithelia, making them prone to the genetic and epigenetic changes for tumor formation [105]. Most of the reports provide evidences toward the role of multiple molecular alterations (mutations in oncogenes, loss of heterozygosity, genomic instability and microsatellite alterations, and TSG along with deregulation of the telomerase activity) in field cancerization [147].

1.2.1 Cellular Basis of Field Cancerization

The cellular basis of field cancerization is explained by two main schools of thought: polyclonal mode and the monoclonal mode of origin. Although the complete basis of these models is yet to be established, existing evidences do point out to both these theories being plausible. The classical model for the origin of field cancerization is the "polyclonal model" suggesting that the multifocal carcinomas developing in the region are of independent origin through mutations occurring in multiple sites of the epithelium due to continuous carcinogen exposure [106]. The tumors, thus originating, though are in the adjacent fields, will be genetically

different and are hence polyclonal. Studies in pancreatic and colon cancers suggest a polyclonal origin for the multiple lesions that develop in a patient owing to the distinct K-Ras mutations observed in each lesion [107, 108]. An initial study in head and neck cancer by profiling of p53 and its downstream proteins indicated that simultaneous, preinvasive and invasive lesions in patients showed distinct molecular profile [109, 110].

The monoclonal origin of the field wherein the lesions share a common clonal origin and develop due to migration of the cells from the initial lesion is the second concept of cancerization. Experimental evidences do point out to the feasibility of this model also in bladder cancer, though the underlying basis is not delineated [107, 111]. In order to explain the possible mechanisms driving this concept, three theories have been postulated (Fig. 1.3). *The first theory* suggests that tumor cells or tumor progenitor cells migrate through the submucosa to another site (intraepithelial migration). *The second theory* implies that cells shed into the lumen of an organ (primary tumor site) form the tumor in a secondary site. *The third and the final proposed theory* is based on findings that the genetically altered field in the epithelium originates from clonally related neoplastic lesions that develop *via* lateral spreading in same or adjacent anatomical areas. The final theory also justifies the presence of the patch-field model, wherein a large area of normal mucosa is replaced by genetically altered preneoplastic cells awaiting the second hit to progress to tumorigenic state (Figs. 1.6, 1.7) [103, 104, 106, 112–115].

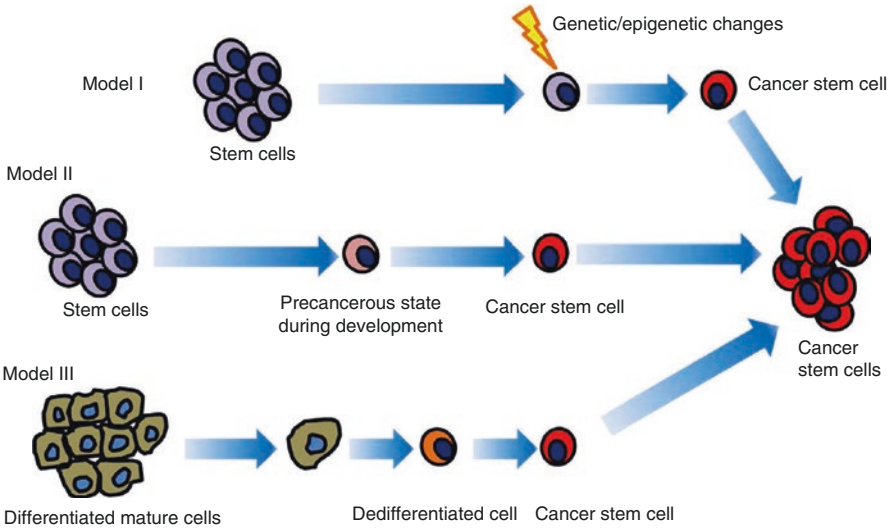


Fig. 1.6 Three possible mechanisms of development of cancer stem cells (Need to get reprint approval from Mohanta et al., 2015 [154])

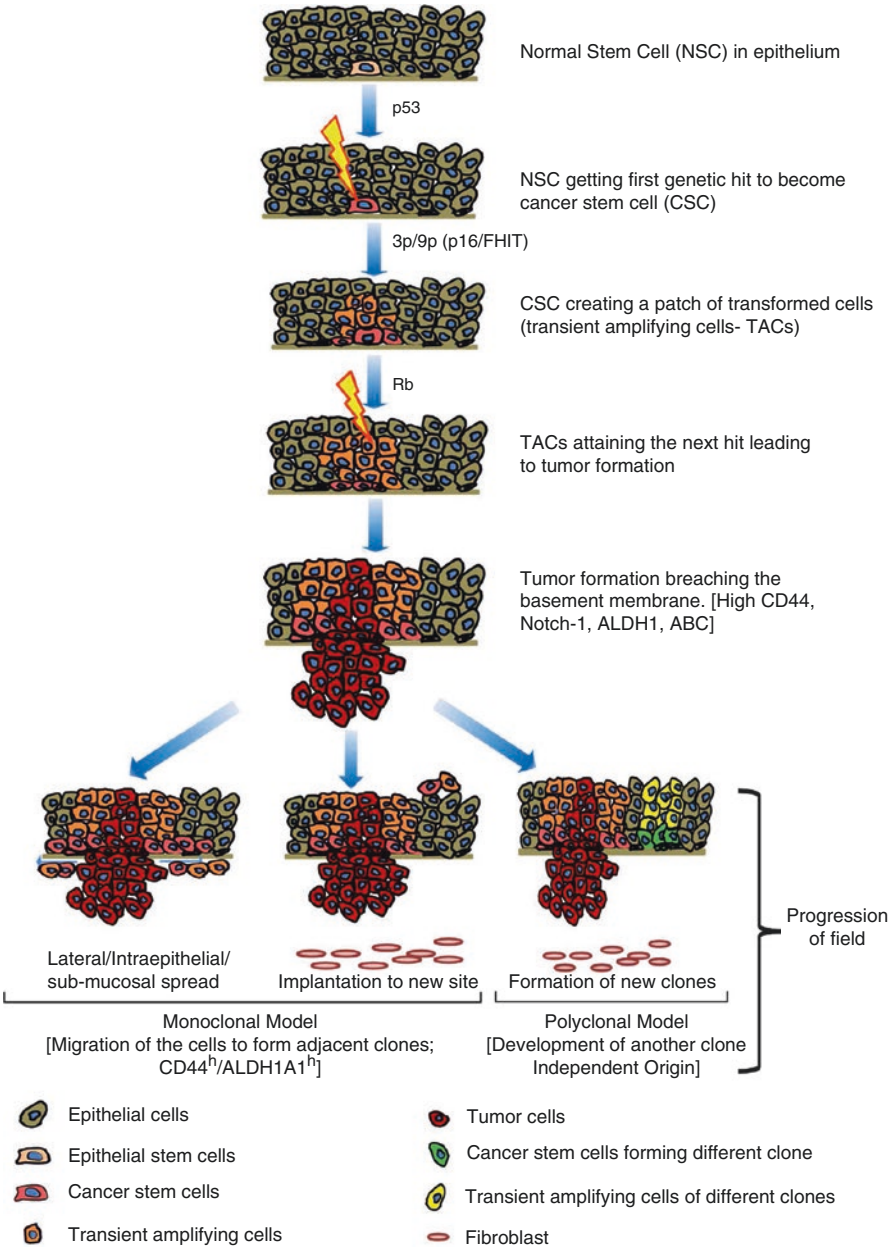


Fig. 1.7 Possible mechanisms of development of field cancerization (Need to get reprint approval from Mohanta et al., 2015 [154])

1.2.2 Molecular Models of Field Cancerization

Multiple models of genetic abnormalities underlying field cancerization have been proposed based on the experimental evidences accumulated down the decades. An initial model proposed by Brakhuis et al. in 2003 [116] is based on the genetic alterations associated with the stepwise histological progression observed in carcinogenesis of HNSCC. The model proposed that the transformation of a normal epithelium to a cancerous one initiates TP53 (17p) mutations in cells which ultimately lead to the development of a patch, consisting of a clonal unit of *these* cells. Subsequently, the patch converts to a field, which is an epithelial lesion consisting of cells with successive cancer-related genetic alterations. This field eventually replaces the normal tissue, and during field progression, additional genetic mutations occur in chromosomes 3p, 9p, 8p, and 18q. With the mutation in 11q12, the field is suggested to transform to a carcinoma *in situ*. During field progression, additional genetic mutations occur in chromosomes 3p, 9p, 8p, and 18q. With the mutation in 11q12, the field is suggested to transform to carcinoma in situ.

Califano et al. have also described the genetic progression model of field cancerization in head and neck cancer. According to this model, the transformation of the normal mucosa is initiated by hits to the 9p region leading to the development of benign hyperplasia. This lesion then further progresses to dysplasia by successive mutations in the 3p and 17p region, with the modifications in 17p region suggested to drive the development of the first patch of mutated neoplastic cells. The patch further expands into the field, which then transforms to cancer with mutation in 11q and 13q chromosomes (18) (Fig. 1.7).

1.2.3 Biomarkers of Field Cancerization

Studies to understand the molecular basis of field cancerization have led to the identification of number of biomarkers that can, ideally, determine the abnormal "field" of transformed cells that are either inherited or arise due to continuous and sustained carcinogenic assault. Detection of these markers in the histologically normal mucosa surrounding the primary tumor is suggested to be indicative of the extent of the field. Molecular markers such as loss of heterozygosity (LOH), microsatellite alterations, telomerase activity, chromosomal instability, and mutation in TP53 gene are some of the established means to distinguish and characterize these resident cells of the "field" that develop during the cancerization process [112, 114, 115, 117–119].

1.2.3.1 TP53 Mutations

p53 overexpression is a common event in head and neck cancers with the protein being involved in the maintenance of cellular integrity caused due to DNA damage. Suppression or alteration of p53 pathway is known to lead to genomic instability and trigger carcinogenesis in head and neck cancer [120]. Assessment of the TP53 status in adjacent normal mucosa provides evidence toward it being one of the early changes that initiate the process of cancerization. Study on patients with oral cancer

showed the presence of p53 mutations in the surgical margins correlated with their clinical outcome [121–123]. On the basis of p53 mutations, Brakhuis et al. classified the tumor as primary tumor, second primary tumor, and recurrent tumor [116, 124] indicating that molecular changes in p53 can be indicative of the clonal origin of the tumor. Mutated p53 was thus considered indicative of molecularly "premalignant cells" in a histologically normal oral mucosa [125].

1.2.3.2 Loss of Heterozygosity

Loss of heterozygosity (LOH), indicating the loss of allelic material adjacent to microsatellite markers, is another marker used to study the clonality of premalignant lesions in the adjacent normal mucosa of the tumor. LOH at different chromosomal locations is known to be an established marker of field cancerization in the normal mucosa. Short tandem repeat (STR) markers specific to the regions 3p12, 3p14, 3p21, 9p21, 9p22, 17p13, and 13q14 have been used to identify molecularly abnormal cells in the tumor-adjacent mucosa. Among these markers, evidences in oral cancer show that LOH at 9p21 is detected in histologically normal mucosa, while changes at 3p accompanied dysplastic changes [126]. Abnormalities at 3p and 9p along with 17p have also been used to distinguish the clonality between the multiple invasive and preinvasive lesions in patients with oral cancer [127].

1.2.3.3 Telomerase

Telomerase levels are enhanced in transformed cells as an attempt to achieve immortalization; the presence of this enzyme in the tumor-adjacent mucosa can also be a relevant marker of cells that are transformed at the molecular level. Studies of telomerase activity in sample cohorts that included adjacent mucosa precancerous and cancerous lesions showed high enzyme activity in 30–70 % of normal tissue [61, 128]. This was suggested to be due to the increased tobacco usage in the patient cohorts further emphasizing that concept of the "abnormal field." The assessment of telomerase status by the TRAP assay (telomerase repeat amplification protocol) in the normal mucosa of oral cancer also showed increased levels in the sample that was predictive of recurrence [129]. Similar studies in cancers of other sites such as breast tumor also showed the presence of hTERT expression in histologically normal tissue [122].

1.2.3.4 Ploidy Analysis

Ploidy analysis, which documents the DNA content in the cells, has also been used as a technique to detect abnormal cells in the tumor-adjacent mucosa. Studies in the oral mucosa of the hamster cheek model, have reported that tissues with no atypical dysplastic changes, have been identified to have abnormal DNA content indicative of the cancerization process [130, 131]. Chromosomal polysomy in various grades of dysplasia was also indicative of field cancerization in patients with head and neck cancer [132]. Evaluation of the DNA index (DI) quantifying the diploid, aneuploid status of the cells in oral premalignant and the normal-appearing mucosa has been identified as a highly significant risk factor [133, 134]. Multiple genomic aberrations

at 20q13, 7p22, 11p15, and 16p13 were also identified to be common between the non-dysplastic mucosa and the dysplastic oral lesions [135, 136]. Another study also pointed out that abnormal changes at chromosomes 7 and 17 were significantly different between tumor-adjacent and tumor-distant mucosae, which were also observed in increasing frequency in the different grades of dysplasia [137, 138].

1.2.3.5 Angiogenesis Markers

Nuclear organizer regions and subepithelial vascularization in the tumor-adjacent mucosa are considered accurate markers of abnormal alterations that precede the histological changes [139]. Gazzar et al. have also reported significantly higher vascularity index in tumor-adjacent normal oral mucosa as compared to the mucosa of non-cancer patient. Vascularization as detected by CD31 and VEGF expression, has also been detected in the normal mucosa along with the dysplastic and the non-dysplastic premalignant lesions [42, 140].

1.2.3.6 Other Markers

Studies to assess the molecular changes that characterize the normal, tumor-adjacent mucosa and thereby indicative of field cancerization have identified several other markers. Expression of cytokeratins (CK19, 8/18, 19), MMPs (MMP 9), and growth factors (EGFR, TGF) in adjacent normal mucosa of the tumor have been identified as markers of field cancerization [141, 142]. Expression patterns of MIB1 and Cyclin D1 were significant for determining the field cancerization [143]. Dysregulated expression of adhesion molecules such as CD44, cadherin, and β-catenin is also suggested to be indicative of neoplastic progression in the tumor-adjacent mucosa [144].

1.2.4 Cancer Stem Cells in Field Cancerization

Cancer stem cells (CSCs), named for their potential to give rise to tumors, are tissue specific and can migrate, properties that provide support to the concept of these cells being the underlying basis of field cancerization. In the oral mucosa, wherein the differentiated epithelial cells have a high renewal rate (every 14 days) [145], the long-time residents of epithelium, the slowly dividing, oral stem cells (SCs), are more likely to accumulate the necessary hits mandatory for transformation. The detection of CSC markers in the tumor-adjacent normal mucosa provides evidence toward their role in cancerization. In OSCC, studies have revealed that tumor-adjacent normal tissues of recurrent and the non-recurrent patients showed expression of CSC markers such as ATR, CD44, ABCG1, and ANKRD50 [146, 147]. Studies in rat oral carcinogenesis models have shown an expression of SC-related markers such as Oct4 and Sox2 in the normal and transforming oral mucosa. These results were further validated when these markers were expressed in the non-cancer tissue adjacent to the tumor and in the precancerous lesions of oral cancer patients [148].

Similar evidences are available in other tumors also; single and multiple clonal tumors with CSC markers have been reported in the gastrointestinal tract. It has been reported that normal human gastric stem cells can acquire mutations, proliferate, and ultimately lead to the formation of a new patch of abnormal cells in the preneoplastic field [149, 150]. Injury to lung tissue is also reported to lead to a deregulated repair of stem cells, which then form a clonal group of indefinitely self-renewing daughter cells in the normal mucosa. Additional mutations lead to proliferation and finally result in a stepwise progression of the disease in the tissue [151]. Studies in breast cancer samples also provided a clinical correlation; CD44+/CD24+ cells were enriched in the adjacent mucosa of patients with triple-negative breast cancers indicating a possible prognostic significance [152, 153]. A recent review from our lab has comprehensively cataloged the possible implications of CSCs in field cancerization. A multitude of CSC-markers have been associated with the various processes involved in field cancerization (Table 1.3).

Table 1.3 CSC-related markers that could aid in detection of field cancerization [154]

Types of marker	Marker	Cancer stem cell relation	Role in field cancerization	Detection in adjacent mucosa
Pluripotent markers	Oct4	Cancer stem cell marker in oral cancer; associated with prognosis	Dedifferentiation of tumor/mature cells	✔
	Sox2	SOX2 has role in regulating cancer stem cell properties of pancreatic cancer cells	Dedifferentiation of tumor/mature cells; tumor Initiation	✔
	Nanog	Moon et al. have reported that Nanog has a role in genesis of cancer stem cells in GBM	Dedifferentiation of tumor/mature cells	✘
Aldehyde dehydrogenase	ALDH1A1	ALDH1+/CD44+ cells show increased migration and tumor initiation	*Intraepithelial migration, tumor initiation*	✘
Drug transporter	ABCG2	Stem cell marker imparting drug resistance in HNSCC; ABCG2+ cells increased tumor initiation	*Tumor initiation/ drug resistance*	✘
Adhesion molecule	CD44	CSC marker in HNSCC	*Tumor initiation*	✔
	CD133	Putative CSC marker in brain, prostrate, and head and neck cancer	*Tumor initiation*	✘

(continued)

Table 1.3 continued

Types of marker	Marker	Cancer stem cell relation	Role in field cancerization	Detection in adjacent mucosa
EMT markers	E-cadherin	Marker of EMT and CSCs (breast cancer spheroids positive for E-cadherin)	*Epithelial migration*	✔
	S100A4	Putative CSC marker in HNSCC	*Epithelial migration*	✔
	MMPs	Implicated in the invasive behavior of CSCs in colorectal cancer and OSCC	*Epithelial migration*	✔
	SNAIL	EMT marker that maintains self-renewal properties of CSCs	*Tumor initiation/ migration*	✔
	S100A8	Progression of disease in colorectal carcinoma and migration of cancer stem cells	*Epithelial migration*	✔
Tumor supressor genes/ oncogenes/ cell cycle regulatory gene	Cyclin D1	Induces EMT in CSCs in ovarian cancer	*Epithelial migration*	✔
	K-Ras	Mutations in K-Ras activate CSCs contributing toward tumorigenesis as well as metastasis in the cells	*Tumor initiation*	✔
Differentiation antigen	CK8/18, CK19	CK8/18 is expressed CSCs of papillary carcinoma; CK19 in cutaneous epithelial lesions	*Proliferation/ initiation*	✔
	Telomerase	Telomerase enzymatic blockers, such as Imetelstat, have been shown to decrease CSC populations	*Tumorigenesis*	✔
	RAR	Expression correlates with CSC expression in pancreatic cancer	*Tumorigenesis*	✔
Proliferation marker	Ki67	Ki67 is a marker of cancer stem cell of glioblastoma	*Proliferation*	✔
Growth factors/ receptors	EGFR	EGFR is highly expressed in CD133 positive glioblastoma	*Tumorigenesis*	✔
	VEGF	EMT-induced VEGF-A expression can lead to tumorigenesis	*Angiogenesis/ tumor initiation*	✔
Drug-resistant genes	ATR	Inhibition of ATR abrogates tumorigenicity of colon cancer cells through depletion of CD133 positive cancer stem cell population	*Drug resistance*	✔

Assessment of the role of the cancer stem cells in field cancerization process thus gives rise to a new concept that can further be employed toward identification of novel markers. The concept, if established, can enable identification of predictors of neoplastic transformation in the histologically normal, tumor-adjacent mucosa that can be evaluated for clinical utility.

1.2.4.1 Implication of Field Cancerization in Diagnosis and Therapy in Oral Cancer

In the current scenario, there is a lack of prognostic biomarkers that can predict recurrence and formation of second primary tumor in HNSCC. The extensive marker repertoire that was identified down the decades has neither been used clinically in the assessment of surgical margins nor toward accurate prediction of disease recurrence in HNSCC patients. The most effective way of confronting the disease relapse is accurate prediction of transforming clonal events in surrounding normal epithelium at the time of cancer resection. Knowledge of early events of carcinogenesis can be used to identify residual clonal populations in tumor margins by molecular analysis to more accurately assess the successful surgical resection.

The concept of field cancerization implies cause-effect reasoning for the generation of secondary and recurrent tumors. Future research focus should be on identification of molecules and molecular events that affect prognosis. New tumor markers have yet to be clinically applied with the ultimate goals including the prevention and effective treatment of head and neck cancer. Delineating the role of cancer stem cells in field cancerization would provide a different approach toward understanding of their role in the progression of the disease, besides providing cues for developing novel treatment modalities targeting these cancer stem cells. By using these tumor-initiating cell signatures as biomarkers on progenitor cells, postsurgical state of patients can be known, which can customize the therapeutic regime and improve the efficacy of current cancer therapies.

1.2.4.2 Definition of Terminology

Loss of Heterozygosity and Single Nucleotide Polymorphism

Most diploid human somatic cells contain two copies of the genome, one from each parent (chromosome pair). Each copy contains approximately 3 billion bases (adenine (A), guanine (G), cytosine (C), or thymine (T)). For the majority of positions in the genome, the base present is consistent between individuals; however, a small percentage may contain different bases (usually one of two, for instance, “A” or “G”), and these positions are called “single nucleotide polymorphisms” or “SNPs.” When the genomic copies derived from each parent have different bases for these polymorphic regions (SNPs), the region is said to be heterozygous. Most of the chromosomes within somatic cells of individuals are paired, allowing for SNP locations to be potentially heterozygous. However, one parental copy of a region can sometimes be lost, which results in the region having just one copy. The single copy cannot be heterozygous at SNP locations, and therefore the region shows loss of heterozygosity (LOH). Loss of heterozygosity due to loss of one parental copy in a region is also called hemizygosity in that region.

Microsatellites It is also known as *simple sequence repeats* (SSRs) or *short tandem repeats* (STRs). They are repeating sequences of 2–5 base pairs of DNA. They are unique to an individual or a tumor. It can be used as molecular markers in STR analysis, for family, population, and tumor clonality. They can also be used for studies of gene duplication or deletion, marker-assisted selection, and fingerprinting.

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Aetiology of Oral Cavity Cancer

2

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2.1 Introduction

Oral cancer is a serious and growing health problem in many parts of the globe. Most head and neck cancers are squamous cell carcinomas, and the majority are oral squamous cell carcinoma (OSCC) [1, 2]. The origin of OSCC is oral keratinocytes, and it is caused, as any other cancer, by DNA mutations which may be spontaneous but increased by exposure to a range of mutagens that could be chemical, physical or microbial. Cells with genetic mutations can progress from normal cells to pre-malignant or potentially malignant cells that have the ability to proliferate in a less-controlled fashion than normal [2]. Consequently, the cells become autonomous and cancer results.

2.2 The Theory of Carcinogenesis (See Vol. I, Chap. 1 for Details)

Carcinogenesis is considered as a multistep process with subsequent stages of initiation, promotion and progression. Initiation is an irreversible, non-lethal genetic change that may be hereditary or acquired by an insult from environmental agents, such as chemical carcinogen or oncogenic microbes such as human papillomavirus (HPV) [3, 4]. These genetic alterations occur mainly in regulatory genes such as proto-oncogenes, tumour suppressor genes (TSG) and genes involved in apoptosis and DNA repair [5]. Proto-oncogenes are cellular genes involved in normal cell

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growth and differentiation [6]. They encode growth factors, growth factor receptors and proteins involved in signal transduction and transcription as wells as cyclin and cyclin-dependent kinase. Mutations in these genes may result in transition of proto-oncogene to an oncogene, whose expression and/or product is not influenced by regulatory elements [5].

In contrast, TSG encodes components that suppress cell proliferation. Inhibition of both alleles of a TSG can lead to aberrant cell growth, a key event in carcinogenesis [5]. Further, genetic alterations in genes encoding components involved in apoptosis or DNA repair ultimately facilitate persistence and replication of mutated cells. This initiation (mutation) is followed by a promotion step, which is the accumulation of the genetic changes that can stimulate transformation of normal cells into neoplastic cells. These cells may then form malignant tumours which are characterised by excessive cell growth, invasiveness and metastasis [7]. Philip et al. (2004) [8] suggested a hypothesis on how promotion, such as chronic inflammation, affects carcinogenesis. They hypothesised that initiated cells accumulate during life and that promoters act to (i) cause these cells to accumulate more mutations (possibly by preventing apoptosis of carcinogen-damaged cells and through reactive oxygen formation), (ii) drive these mutant cells to proliferate and (iii) give preneoplastic and neoplastic cells a growth advantage through 'Darwinian selection' [8, 9]. The initiators are usually DNA-reactive substances (carcinogenic mutagens), whilst promoters are usually non-DNA reactive. A single exposure to mutagens may be sufficient to cause cancer, whilst promoters must act over time for tumours to develop, eventually progressing to cancer [8].

2.3 Epidemiology of Oral Cancer (See Vol. I, Chap. 4 for More Details)

Oral cancer is recognised as the sixth most common cancer worldwide, with recent epidemiological data reporting 263,900 new cases of lip and oral cavity cancer in 2008 [10–12]. Globally, there is a wide geographical variation in the incidence of oral cancer. The highest incidence rates of this cancer (excluding lip) are found in the South and Southeast Asia (e.g. Sri Lanka, India, Pakistan and Taiwan), parts of Western (e.g. France) and Eastern Europe (e.g. Hungary, Slovakia and Slovenia), parts of Latin America and the Caribbean (e.g. Brazil, Uruguay and Puerto Rico) and in the Pacific regions (e.g. Papua New Guinea and Melanesia) [10].

In Australia, a total of 60,826 new cases of lip, oral cavity and oropharyngeal cancer were diagnosed over the period between 1992 and 2008, representing 2.9 % of the total cancer load in Australia and 1.6 % of all cancer deaths [12]. The lip followed by the tongue continued to represent the most common sites of new oral cancer cases [12]. Even though a decline in the incidence of oral cancer was noticed in Australia in the past three decades, no significant change was observed in the

mortality rate, highlighting the continuous need for early detection and preventive strategies to reduce the incidence of such an avoidable disease [12].

In most countries around the world, oral cancer is more frequent in males than females. This may be due to differences in tobacco and alcohol consumption, dietary intake, sexual behaviours and treatment-seeking practices. However, this trend seems to be changing recently in some parts of the world with reports of comparable incidence rates in males and females [1]. For example, a meta-analysis from Buenos Aires showed the male to female ratio is 1.24:1 for the period 1992–2000 compared to 7.1:1 for the period 1950–1970 [13]. Further, in some regions of India, where betel quid/areca nut chewing is more common amongst women, the incidence of tongue and other intra-oral cancer for women is equal to or greater than that for men [1].

The risk of developing oral cancer increases with age, with the majority of cases occurring at or after the fifth decade of life [10, 14–16]. Recently, there has been a trend of rising incidence particularly related to cancer of the tongue and mouth in young patients. It is estimated that 4–6 % of oral cancers now occur at ages younger than 40 years [14]. Many studies have reported that traditional risk factors such as smoking and alcohol consumption, considered significant aetiological agents in older patients, were also present in varying degrees in young people. However, there is a dispute whether these are contributory factors in young people at all owing to the relatively short time frame of exposure. Furthermore, other studies provide evidence that many young patients have never smoked or consumed alcohol [14]. These findings suggest that factors other than tobacco and alcohol may be implicated in the development of oral cancer in these patients. It is important to note that, in developed countries, the incidence of smoking has significantly reduced over the past two to three decades, whilst the incidence of oral cancer has not diminished. The data from the United Kingdom probably illustrates this most clearly, with an obvious increase in the European Age-Standardised Incidence Rates per 100,000 Population for Oral Cancer (C00-C06, C09-C10, C12-C14) observed most notably in males, but also occurring in females, from 1975 to 2011 by sex in Great Britain (http://www.cancerresearchuk.org/cancer-info/cancerstats/types/oral/incidence/uk-oral-cancer-incidence-statistics#trends). The information regarding cigarette smoking prevalence in the same population, over this extensive period of time, showed that as a percentage across the whole population, smoking had more than halved in both males and females (http://www.cancerresearchuk.org/cancer-info/cancerstats/causes/tobacco-statistics/#trends).

For most countries, the overall 5-year survival rate for oral cancer is around 50 % for all anatomical sites and stages [10]. TNM (T, tumour size; N, lymph node involvement; M, distant metastasis) stage at presentation significantly affects survival rate. A survival rate of 66–85 % was reported in cases that present without regional lymph node involvement (Stages I and II), whilst in cases associated with lymph node infiltration (Stages III and IV), survival drops to 9–41 % [17]. Therefore, there is a continuous need for early detection and management of oral cancer at earlier stages to improve the survival rates and to decrease the morbidity associated with the treatment of oral cancer lesions.

2.4 Oral Potentially Malignant Disorders (See Vol. I, Chap. 10 for More Details)

The World Health Organisation has recently recommended abandoning the distinction between potentially malignant lesions and potentially malignant conditions and rather to use the term potentially malignant disorders instead [18]. As yet, there is no reliable marker to predict the malignant transformation in an individual patient [2].

Potentially malignant conditions were previously defined as generalised mucosal disorders in which there is a small increased risk of development of malignant or pre-malignant lesion. Atrophy of the oral epithelium is a linking feature in these lesions, which may eventually increase the susceptibility of oral epithelium to carcinogens. Therefore, avoiding exposure to known risk factors should be stressed in these patients. Lichen planus, chronic iron deficiency anaemia and submucous fibrosis are examples of these conditions [19].

Oral potentially malignant lesions (OPML) are considered as clinically recognisable and morphologically altered abnormalities of oral mucosa. They vary from small well-defined white or red mucosa patch to widespread and extensive involvement of the oral mucosa [19]. Clinically, these lesions may present in three forms: leukoplakia, which are white or yellow brown keratinising lesions that are the most frequently encountered and have a comparatively low rate of malignant progression (4–18 %) [20, 21]; erythroplakia (velvet red lesions); and proliferative verrucous leukoplakia. Lesions that are partly red and white are sometimes referred to as speckled leukoplakia. Both erythroplakia and speckled leukoplakia, although they are rare, have been shown to have a high risk of malignant progression (14–50 %) [21, 22].

2.5 Clinical Presentation of OSCC and OPML

Unfortunately many oral carcinomas are entirely asymptomatic; therefore, clinicians must be aware of the clinical presentation of oral malignant and pre-malignant lesions as failure to recognise these lesions can lead to treatment delay or poor outcomes. Oral cancer may present as solitary oral lump, ulcer, white or red patch persisting for more than 3 weeks or non-healing socket. Unexplained loose tooth or pain or numbness of the tongue should also cause concern until proven otherwise. Other common presentations include abnormal tongue movements, sudden poor fit of dentures, alterations of speech, neck swelling and obstructive disease of the submandibular glands from carcinoma in the floor of the mouth [2, 19].

Scalpel biopsy is invariably indicated to confirm a clinical diagnosis, and if a biopsy is negative in the light of strong clinical suspicion, then it is important to consider repeated biopsy/investigation until diagnosis is established or refuted [2, 19]. Since visualisation is the principal strategy to find patients at risk of oral carcinoma, a number of adjunctive diagnostic tools have been used to aid in the early detection of oral cancer lesions. These visual tools include the use of vital staining

(e.g. toluidine blue) and optical techniques such as ViziLite™, VELScope™, Identafi™ and Narrow Band Imaging [23–30]. Evidence for their use and efficacy as adjunctive aids is gathering although they do not at present represent an alternative to a thorough oral examination and conventional histopathology of a mucosal biopsy [31].

2.6 Risk Factors for OSCC

The aetiology of oral cancer is multifactorial. The vast majority of oral squamous cell carcinoma has been related to tobacco usage in various forms and heavy alcohol drinking, but other factors such as areca nut/betel quid chewing, radiation exposure, infections, immunoincompetence and dietary intake may be relevant in some cases. Many of these factors are related to lifestyle, but environmental and genetic factors may play roles. The most relevant factors are discussed below.

2.6.1 Tobacco

Tobacco use is by far the most widespread link between exposure to known carcinogens and death from cancer [32] and continues to be the leading global cause of preventable death [33]. Smokers lose at least one decade of life expectancy, as compared with those who have never smoked [34]. Tobacco kills approximately six million people and causes more than half a trillion dollars of economic damage each year [33].

2.6.1.1 Cigarette and Cigars

Since the 1920s, most smoking tobacco has been consumed in the form of cigarettes although there is a wide variety of smoking tobacco products available around the world including cigars, cigarillos, bidis, chuttas and kreteks. A cigar is any roll of tobacco wrapped in leaf tobacco. Roll-your-own (RYO) cigarettes are a cheaper substitute for commercially manufactured brands and are gaining in popularity worldwide. Both tobacco and tobacco smoke are very complex matrices consisting of thousands of compounds. There are more than 60 carcinogenic combustion products in tobacco smoke. The general concept of exposure to carcinogens, metabolism to reactive intermediates and DNA damage leading to mutations in critical genes has been established as one major mechanism by which tobacco smoke causes cancer [35]. Nicotine, not in itself carcinogenic, is toxic and addictive. Its resultant addiction, however, promotes continued use of tobacco products which contain many carcinogens, in particular strong carcinogens such as polycyclic aromatic hydrocarbons (PAHs), nitrosamines and aromatic amines and weak carcinogens such as acetaldehyde. The activity of these carcinogens is largely exerted through DNA adducts [32]. Some constituents of tobacco smoke, or their metabolites, may bind directly to cellular receptors, leading to activation of protein kinases, growth receptors and other pathways, which can contribute to carcinogenesis [36].

Numerous case–control cohort studies have confirmed a key role of tobacco smoke in human cancer, and the risk is strongly dose dependent with multiplicative synergism seen with alcohol drinking [37, 38]. Hashibe (2009) [38] found that the population attributable risk (PAR) for tobacco or alcohol was 72 % (33 % tobacco alone, 4 % alcohol alone and 35 % was due to tobacco and alcohol combined). The total PAR differed by subsite (64 % for oral cavity cancer, 72 % for pharyngeal cancer, 89 % for laryngeal cancer). Hashibe (2013) [39] reported on another cohort where the proportion of head and neck cancer cases attributed to tobacco and/or alcohol was 66 % (50.5 % tobacco alone, 14.7 % alcohol alone, 0.9 % tobacco and alcohol combined). Thus, the overwhelming role of smoking in the aetiology of oral cancer needs to be questioned. In a recent report from the Netherlands Cohort Study where an initial 120,652 participants were enrolled in 1986, they demonstrated the incidence rate ratio for head and neck cancers, using Cox proportional hazards model after 17.3 years of follow-up, during which time 395 individuals were diagnosed with head and neck cancers, 110 oral, 83 oro-/hypo-pharyngeal, and 199 laryngeal cancers [40]. This data clearly indicates for the first time a much greater risk for the development of oral cancer with alcohol consumption (RR = 6.4; 95 % CI 3.1–13.0) than with smoking (RR = 2.1; 95 % CI 1.2–3.6) [40]. The associated risk of this behaviour for pharyngeal and laryngeal cancers is almost directly the opposite, with very high risk associated with smoking for pharyngeal (8.4) and laryngeal (8.1) and much lower for alcohol consumption (3.5 and 1.5, respectively) [40]. Thus, it would appear that with a decrease in cigarette smoking, a true association between alcohol consumption and oral cancer is now emerging.

2.6.1.2 Smokeless Tobacco

Smokeless tobacco includes a large, worldwide variety of commercially available or home-made products and mixtures that contain tobacco, whether it is in leaf form or pulverised into a powder, as the principal constituent and are used either orally or nasally without combustion. Oral smokeless tobacco products are placed in the mouth, cheek or lip and sucked (dipped) or chewed [41].

Chewing tobacco includes loose leaf, plug and twist tobacco [42]. Loose-leaf chewing tobacco is used primarily by men in the United States, commonly in conjunction with outdoor activities, where the resulting tobacco juices can be expectorated. Dry snuff is a fermented, fire-cured tobacco that is pulverised into powder, and its original use was through nasal inhalation. The popularity of these products has waned, with consumption declining gradually over the past century [43]. Moist snuff is the only tobacco product in the United States with increasing sales [42]. Traditional moist snuff users place a ‘pinch’ of the finely ground tobacco between the gingiva and buccal mucosa. Modern moist snuff products are sold in small, preportioned pouches similar to teabags [43]. The use of snus (Swedish moist snuff) was found to be a significant factor in the low prevalence of smoking, especially amongst younger men and women in Northern Sweden [43]. At least 16 carcinogens are found in chewing tobacco [32]. In particular, tobacco-specific *N*-nitroso compounds have been detected at high concentrations in both snuff and chewing tobacco [44]. Additionally, tobacco (and quid chewing) may cause oxidative stress

to tissues through the release of reactive oxygen species, which initiate free radical reactions, causing damage of protein, lipids, carbohydrates and DNA [45].

Rodu et al. concluded that the use of dry snuff is associated with a higher relative risk (RR = 4–13), unspecified type smokeless tobacco was associated with an intermediate relative risk (RR 1.5–2.8), whereas chewing tobacco and moist snuff was associated with very low risks for cancers of the oral cavity and related structures (RR 0.6–1.7) [43].

Scandinavian moist snuff (snus) is claimed to be a safer alternative to smoking [46]. It was suggested that an increased risk of oropharyngeal cancer is evident most clearly for past smokeless tobacco use in the United States, but not for Scandinavian snuff [47]. This has been suggested to be because Swedish snus is not fermented and contains much lower nitrosamine levels than fermented tobaccos [48]. In contrast, a population-based prospective study provided suggestive evidence of snus-related risks including statistically significant increase in the incidence of the combined category of oral and pharyngeal cancer amongst daily users of snus [46].

The term 'snuff-induced lesions' is sometimes used in research articles, but is ambiguous in its definition. Risk increases with increasing length of exposure, with risk greatest for anatomic sites where the product is held in contact for the longest time [49]. For example, a study by Little et al. reported that 78.6 % of daily smokeless tobacco users (two-thirds using moist snuff and one-third using chewing tobacco) had oral lesions in contrast to 6.3 % of non-users of ST [50].

2.6.1.3 Waterpipe Smoking (aka Hookah, Sheesha, Shisha and Narghile)

The waterpipe, referred to as hookah, shisha or narghile in different cultures, is a centuries-old tobacco use method, traditionally in Middle Eastern societies [51]. Since the 1990s, there has been a re-emergence of popularity, particular amongst youth in the Eastern Mediterranean Region, but also all over the world [52]. Available evidence suggests that the prevalence of current (past month) waterpipe smoking ranges from 6 to 34 % amongst Middle Eastern adolescents and 5 to 17 % amongst American adolescents, with its use on the increase [53]. The most popular type of waterpipe tobacco is called maassel (also known as shisha tobacco), a wet mixture of tobacco, sweetener and flavourings. There are any flavours, including fruit and candy, producing an aromatic smoke that may be particularly appealing to youth [54]. Misconceptions about waterpipe smoke content may lead users to underestimate health risks [54]. Even if it has been passed through water, the smoke produced contains high levels of toxic compounds [53]. Depending on the toxicant measured, a single waterpipe session produces the equivalent of at least 1 and as many as 50 cigarettes [54].

Research indicates substantial harmful effects of waterpipe tobacco similar to those of cigarettes. Additionally, there is a potential of providing a bridge to cigarette smoking or relapse [53]. For example, a recent systematic review found that waterpipe smoking more than doubles the risk of lung cancer, but was not significantly associated with bladder cancer, nasopharyngeal cancer, oesophageal cancer or oral dysplasia. There is a need for high-quality studies to identify and quantify with confidence all the health effects of this form of smoking [55].

2.6.1.4 Areca Nut/Betel Quid

Areca nut is used as a masticatory substance by approximately 600 million people worldwide [56]. The usage of areca nut is indigenous to India, Sri Lanka, Maldives, Bangladesh, Myanmar, Taiwan and numerous islands in South Pacific and popular in parts of South East Asia and in migrant populations from any of these countries.

The nut may be used fresh, or dried and cured before use, by boiling, baking or roasting or even fermented [56]. Common accompaniments to areca nut include the betel leaf (piper betel), lime (calcium hydroxide, usually from sea shells), catechu and other spices, the mixture referred to as betel quid or pan. Pan Masala is the generic term used for areca nut-containing products that are manufactured industrially and marketed commercially. When the combination includes tobacco, it is called *Gutka*, a preparation commercialised since 1975 [57]. Betel quid is chewed for many reasons, including for its psycho-stimulating effects, to induce euphoria, to satisfy hunger and to sweeten the breath and as a social and cultural practice that is strongly entrenched in people's day-to-day life [58]. Additionally, areca nut use with and without tobacco additives has been significantly associated with dependence syndrome [59].

The use of betel quid is associated with potentially malignant disorders such as oral leukoplakia and erythroplakia, oral submucous fibrosis and oral lichenoid lesions [58]. A large case–control study found that after adjusting for age, sex, education and BMI, those who chewed betel quid without tobacco and were additionally non-smokers and non-drinkers had an odds ratio of 22.2 (95 % CI 11.3–43.7) for oral leukoplakia, 56.2 (95 % CI 21.8–144.8) for oral submucous fibrosis, 29.0 (95 % CI 5.63–149.5) for erythroplakia and 28.3 (95 % CI 6.88–116.7) for multiple potentially malignant lesions [60]. Oral submucous fibrosis is a chronic insidious irreversible disease of the oral mucosa characterised by loss of mucosal elasticity and excessive fibrosis [57, 61]. It is well established as a condition with high malignant potential, with reported risk of malignant transformation varying from 2.3 to 7.6 % [62].

Betel quid without tobacco has an independent positive association with oral cancer [63]. Smokeless (aka chewing) tobacco, often used as a component of betel quid, and betel quid without tobacco are both strong and independent risk factors for oral cancer [63]. Pooled oral cancer odds ratio in a meta-analysis of observational South-East Asian studies for betel quid chewers was 7.9 (95 % CI 6.7–9.3) and those who smoked, drank and chewed was 40.1 (95 % CI 35.1–45.8). Amongst the smoking–drinking–chewing subjects, individual effects accounted for 6.7 % of the risk from smoking, 3.1 % from drinking and 17.7 % from chewing, whilst the interaction effect accounted for the remaining 72.6 % [64]. Betel quid chewing and cigarette smoking patients are more likely to be diagnosed with oral cavity cancer at a younger age than those who have just one habit or none [65]. A retrospective Taiwanese study of 1570 OSCC patients found that despite similar disease severity (tumour depth and nodal involvement) and surgical margins, preoperative betel quid chewers had a higher incidence of local recurrence and second primary tumours than non-chewers. This was suggested to support the concept that betel chewing-associated carcinogens could induce fields of molecular alterations and subsequent susceptibility to local recurrence and new primary tumours [66].

2.6.2 Alcohol

2.6.2.1 Alcohol and Oral Cancer: Epidemiological Evidence

In 2002, the number of people worldwide who regularly consume alcoholic beverages was estimated to be greater than 1.9 billion people. The average daily consumption was approximately 13 g of ethanol (~1 drink) [67]. Of these consumers, 80 million are expected to have diagnosable alcohol abuse disorders [68]. Consumption is believed to be rising in many countries, especially in regions of rapid economic growth and amongst women [69]. The World Health Organisation's global burden of disease project assessed the number of deaths that could be attributed to alcohol in 2000. They found that the global burden of alcohol amounts to 1.8 million deaths per year (approximately 3.2 % of all deaths) [70].

In 2007, the International Agency for Research on Cancer, following a review of the available epidemiological evidence, concluded that 'alcoholic beverages are carcinogenic to humans (Group 1)' and 'the occurrence of malignant tumours of the oral cavity, pharynx, larynx, oesophagus, liver, colorectum, and female breast is causally related to alcohol consumption'. In addition to this, the agency classified the ethanol contained within alcoholic beverages as 'carcinogenic to humans (Group 1)' [67]. Worldwide, approximately 389 000 cases of cancer can be attributed to chronic alcohol consumption (3.2 % of all cancers) [71].

The possibility of alcohol consumption being an independent risk factor for the development of OSCC was first explored in 1961 [69]. Numerous epidemiological studies and reviews since that time have investigated the association [3, 38, 69, 72–87]. By adjusting odds ratios for confounding factors and analysing risk factors in non-smokers, these studies have indeed confirmed the existence of alcohol consumption as an independent risk factor for OSCC. There are several patterns evident. Firstly, increases in risk are strongly exposure (drinks per week) dependent, indicating that there is a significant dose–response relationship [72–81]. Whilst definitions of exposure varied between studies, drinkers with 'high' exposure consistently had higher risk than those with 'moderate' exposure. Excess risk for 'high' exposure varied from 2.2 (>56 drinks/week) to 12.0 (>90 drinks/week) [73, 74]. Whilst this is a significant range of odds ratios, all of the mentioned studies consistently showed some form of dose–response effect. In 2010, Tramacere et al. published a meta-analysis of 42 case–control studies, including 17,085 positive cases, examining alcohol intake and oral and pharyngeal cancers. Here it was found that the pooled relative risk for heavy drinking (≥4 drinks/day) was 5.24 (95 % CI, 4.36–6.30), compared to a relative risk for light drinking (≤1 drink/day) of 1.21 (95 % CI, 1.10–1.33), clearly demonstrating the dose–response relationship [88]. Secondly, there are mixed results regarding the effects of 'moderate' alcohol intake. Studies have variably reported that there was no excess risk generated from moderate intake and that a significant increase in risk occurred [72, 74, 80, 81]. Castellsague et al. concluded that consumption of even one drink a day leads to a significant increase in risk [81]. In their meta-analysis, Tramacere et al. found that one drink or less a day conferred a relative risk of 1.21 (95 % CI, 1.10–1.33) [88]. Similarly large meta-analyses by Bagnardi et al. and Li et al. concluded that one drink or less a day

conferred a relative risk of 1.17 (95 % CI, 1.06–1.29) and 1.26 (95 % CI, 0.94–1.67), respectively [89, 90]. Similarly mixed results were found regarding the effect of the duration of alcohol intake (drinking history), with several studies indicating an increased risk with increased duration whilst others indicated no effect of duration [69, 72, 74, 75, 77, 80, 81, 83, 85–87, 91–93]. With regard to the cessation of alcohol intake, the majority of studies found that cessation leads to an immediate reduction in risk [75, 81, 93]. Castellsague et al. found that there was a significant risk reduction within 3 years; however, it took 14 years to approach the risk of a non-drinker [81]. On the other hand, Francheschi et al. found that risk actually peaked 7–10 years after alcohol cessation and that even 10 years after cessation, there was no reduction in risk [74]. In 2012, a meta-analysis determined that risk declined after cessation, but it took approximately 16 years to eliminate any elevated risk [94]. Studies have also consistently shown that ethanol concentration within beverages acts as an independent risk factor in the development of OSCC [76, 78, 81, 87]. As the ethanol concentration within a beverage increases, so does risk. This was evidenced in the study by Huang et al., who found a 6.4 times increased risk with strong spirits compared to other beverages, even after adjusting for total ethanol intake. It was suggested that this phenomenon may indicate local effects of the ethanol in the oral cavity contributing to carcinogenesis [78].

One of the challenges associated with gauging the effect of alcoholic beverages in OSCC is the frequent presence of smoking as a cofactor. Epidemiological studies are frequently affected by low numbers of non-smoking, heavy drinking participants [86]. As mentioned previously, studies work around this obstacle by adjusting for smoking or concentrating on non-smoking drinkers. An example is the pooled analysis by Hashibe et al., which managed to examine 1072 cases and 5775 controls who were non-smoking drinkers [84]. In 2012, a meta-analysis by Turati et al. including 18,387 positive cases total also attempted to establish alcohol use as an independent risk factor in the development of OSCC by examining non-smokers. It was found that in non-smokers, use of alcohol conferred a relative risk of 1.32 (95 % CI, 1.05–1.67) of developing oral and pharyngeal cancer, whilst heavy drinking increased this risk to 2.54 (95 % CI, 1.80–3.58) [95].

As mentioned previously, the smoking of tobacco products acts as an independent risk factor for the development of OSCC. In addition to this, studies and reviews examining OSCC risk in patients who both smoke and consume alcohol have found that these two factors act synergistically to produce a greater than multiplicative increase in the risk of developing OSCC compared to smoking or drinking alone [3, 68, 72, 75, 76, 80, 81, 87, 96]. A recent INHANCE pooled analysis conducted by Hashibe et al. utilising 12,828 cases of head and neck cancer and 17,189 controls confirmed these results, with the study finding a greater than multiplicative increase in the risk of developing OSCC and pharyngeal SCC when smoking and drinking were present [38]. It has been theorised that this greater than multiplicative effect in the head and neck is brought about by local interaction of tobacco and alcohol leading to potentiation of each other's carcinogenesis [97]. Mechanisms include increased metabolic activation of procarcinogens due to CYP2E1 induction and increased penetration across the oral mucosa. These will be covered in detail in later paragraphs.

2.6.2.2 Molecular Mechanisms of the Genotoxicity of Alcohol in Relation to Oral Carcinogenesis

Whilst there is a positive correlation between alcohol intake and the development of oral cancer, ethanol itself is generally not recognised as a direct carcinogen [69]. However, there are a number of proposed secondary mechanisms by which ethanol indirectly causes genetic damage, thus leading to carcinogenesis.

2.6.2.3 Acetaldehyde

Acetaldehyde is a highly reactive aldehyde that is produced during the breakdown of ethanol. Recently, the International Agency for Research on Cancer decided that there was enough evidence to conclude that acetaldehyde associated with the intake of alcoholic beverages is a Group 1 carcinogen in humans and is causally related to cancers of the oral cavity, pharynx, oesophagus and larynx [98]. There are a number of mechanisms via which acetaldehyde contributes to genetic damage.

Upon entering the body, ethanol is metabolised to acetaldehyde primarily by the enzyme alcohol dehydrogenase (ADH). The intermediate is then removed by aldehyde dehydrogenase (ALDH). Whilst this process primarily occurs in the liver, it has been shown that the required enzymes are expressed in the oral mucosa and gingiva [99]. Certain polymorphisms in these two enzymes predispose towards a build-up and reduced clearance of acetaldehyde. The gene polymorphism ALDH2*2, found in Asian populations, encodes an inactive subunit for the enzyme ALDH2. Heterozygotes who have this allele and the competent ALDH2*1 will have less than 10 % ALDH function and will record threefold higher levels of acetaldehyde in the saliva than a competent homozygote on exposure to ethanol [71, 96]. In a landmark Japanese study, drinking subjects who were heterozygous ALDH2*1/ALDH2*2 and thus had increased acetaldehyde retention were found to have an 11-fold increased risk for oral cancer compared to homozygotes [100]. The increased risk associated with this allele combination has been confirmed by meta-analysis [101]. It has also been shown that drinkers who are heterozygous ALDH2*1/ALDH2*2 have significantly higher levels of markers of acetaldehyde-related DNA damage in their cells compared to drinkers who are homozygous ALDH2*1/1 [102]. Animal studies conducted with ALDH2-knockout mice have shown similar results [103]. Compounding this is the discovery that whilst efficient ADH is expressed in the cells of oral cavity and upper aerodigestive tract, even in competent individuals, the expression of highly active mitochondrial ALDH2 is very low to negligent [99]. These facts suggest that the local build-up of acetaldehyde in cells and saliva results in increased carcinogenetic action in the oral cavity and upper aerodigestive tract, a conclusion that others have also come to [99, 104, 105]. Another enzyme with significant polymorphisms is ADH1B, one of the enzymes responsible for the breakdown of ethanol to acetaldehyde. Individuals who are homozygous for the ADH1B*2 allele demonstrate 40 times more efficient enzyme activity than those who are either homozygous or heterozygous for the ADH1B*1 allele [106]. Studies investigating the significance of these polymorphisms in head and neck cancers found that alcohol drinkers who had the ‘slow’ ADH1B (ADH1B*1/*1 or ADH1B*1/*2) were at significantly higher risk compared to those with the ‘fast’ ADH1B (ADH1B*2/*2)

[107–109]. Whilst this risk profile seems counterintuitive (as acetaldehyde production occurs at a lesser rate), an examination of other sources of acetaldehyde production in the oral cavity provides a possible explanation.

In addition to oral mucosal cells, it has been shown that commensal bacteria within the saliva (particularly oral streptococci) can produce significant amounts of acetaldehyde by utilising bacterial ADH enzymes [104, 110]. Homann et al. found that a chlorhexidine mouth rinse reduced salivary acetaldehyde from an average of 35.3 μM to 21.5 μM during administration of 0.5 g/kg of body weight ethanol (a moderate dose). However, high interindividual variation was observed, and salivary bacterial counts could not be correlated to acetaldehyde production, suggesting that there is high variation between individuals [104]. The production of acetaldehyde by oral flora has been proposed as the reason why poor oral hygiene has been identified as a risk factor for OSCC [71]. This hypothesis is supported by a recent study, which found that a stronger association between poor oral hygiene and head and neck SCC in patients who had a 'slow' ADH1B enzyme compared to the 'fast' allele. The authors proposed that this association is due to the action of bacterial ADHs eclipsing a 'slow' mucosal ADH (resulting in the local production of acetaldehyde), as opposed to the patients with 'fast' alleles where the contribution of bacterial enzymes is less significant [111].

The kinetics of the production of acetaldehyde in the oral cavity has also been investigated. Linderborg et al. conducted an in vivo study that measured salivary acetaldehyde at different time points over 10 min following a sip of a 40 % solution of ethanol. On average, the salivary concentration of acetaldehyde peaked at approximately 180 μM at 2 min after the ethanol challenge. This had declined to approximately 75 μM by the 10 min point as the acetaldehyde was removed [112].

2.6.2.4 DNA Adducts from Acetaldehyde

Exposure of DNA to acetaldehyde leads to the formation of several types of stable adducts (a molecule covalently bonded to a DNA base). These additions can interfere with DNA synthesis and replication, leading to misincorporations and mutations. N^2-ethyl-2′-deoxyguanosine (N^2-ethyl-dG) forms from the reaction of acetaldehyde and deoxyguanosine. Whilst it is the most abundant acetaldehyde adduct formed in DNA, it has been found to have insignificant mutagenic properties in mammalian cells [113, 114]. Whilst N^2-ethyl-dGTP has been found to be readily incorporated into DNA during synthesis, it was only incorporated opposite the correct base [115]. Perrino et al. also discovered that the mammalian DNA polymerase η efficiently bypasses the N^2-ethyl-dG lesion during DNA replication [116]. These facts would account for the low mutagenic potential of this lesion [113, 114]. It has been demonstrated that significant increases in the number of these adducts can be seen in oral keratinocytes 4–6 h after challenge with ethanol [117].

A less prevalent but more sinister adduct than can occur is 1,N^2-propano-2′-deoxyguanosine (1,N^2-PdG) [96]. These adducts are primarily formed from the interaction of croton aldehyde (CrA) and DNA [114]. CrA is an environmental pollutant and by-product of lipid peroxidation that has mutagenic, genotoxic and carcinogenic properties [113, 118]. Whilst originally only shown to occur at extremely

high non-physiological levels of acetaldehyde exposure, Theruvathu et al. discovered that significant 1,N^2-PdG formation could be detected at physiological levels of acetaldehyde exposure (100 μM) when physiological levels of the ubiquitous intracellular polyamine spermidine were present [114]. 1,N^2-PdG has significant effects once adducted to DNA. It leads to the formation of interstrand cross links and DNA–protein cross links and also induces miscoding events with a frequency of up to 12 % [113, 114, 119]. As a result, it has been hypothesised that this lesion is predominantly responsible for the observed genotoxicity of acetaldehyde [113].

2.6.2.5 Chromosomal and DNA Damage by Acetaldehyde

Chromosomal damage in humans can be used as an early biomarker with regard to exposure to genotoxic and carcinogenic agents [120]. One measure that is utilised is the sister chromatid exchange, where two identical sister chromatids exchange genetic information. After the findings of chromosomal abnormalities in the lymphocytes of alcoholics, it was first postulated in 1977 by Obe et al. that it was acetaldehyde and not ethanol that was the agent responsible for causing the aberrations. As a result, Obe et al. showed that it was possible to induce sister chromatid exchanges in mammalian cells at acetaldehyde concentrations as low as 88 μM [121]. This has been confirmed by Helander et al., who found that acetaldehyde induced a dose-related frequency of sister chromatid exchanges at concentrations varying from 100 to 400 μM [122]. Whilst sister chromatid exchange may be viewed as an overly sensitive marker, it can still be used as a conservative estimator of genotoxicity [113].

Evidence exists to suggest that acetaldehyde directly interferes with DNA repair mechanisms, thereby prolonging genetic damage. O^6-methylguanine is a mutagenic DNA adduct that may be produced by exogenous carcinogens inducing alkylation of guanine. Typically, this adduct is removed by the DNA repair enzyme O^6-methylguanine transferase. However, this enzyme was found to be inhibited by acetaldehyde at concentrations as low as 0.01 μM [123, 124]. It is also believed that acetaldehyde binds to and alters the action of glutathione, an important intracellular antioxidant [96].

2.6.2.6 Induction of CYP2E1

The metabolic breakdown of ethanol can also occur via an alternative pathway termed the microsomal ethanol oxidising system (MEOS). This refers to a specific cytochrome P450 enzyme (CYP2E1) that is induced in response to chronic ethanol intake [125]. Induction of CYP2E1 can occur within a week if a 40 g/day (~3 drinks) ethanol intake is maintained; however, this varies between individuals [126]. The proportion of ethanol that is oxidised by CYP2E1 varies, but has been found to be up to 30 % in chronic alcoholics [71]. CYP2E1 induction has been shown to occur in the oral and oesophageal epithelium, indicating that the MEOS is active in these epithelial cells [127–130]. Farin et al. noted that induction of CYP2E1 in oral epithelial cells was greater than that in cells from other epithelial surfaces [127]. Induction of CYP2E1 in the oral tissues has several implications regarding carcinogenesis.

In addition to oxidising ethanol, CYP2E1 can participate in the biotransformation of other compounds. This includes several exogenous procarcinogens that are converted into their active, carcinogenic form by CYP2E1. Examples of these procarcinogens such as N-nitroso compounds (including nitrosamine) and polycyclic aromatic hydrocarbons occur in tobacco smoke [71, 96, 97, 127, 130–132]. For example, Farinati et al. found that oesophageal mucosa from rats fed on a chronic ethanol diet had a significantly enhanced capacity for transformation of the tobacco smoke procarcinogen N-nitrosopyrrolidine compared to controls [131]. This presents one possible mechanism for the synergistic interaction between smoking and ethanol intake with regard to OSCC.

Retinoic acid and its precursor retinol are forms of vitamin A that have important effects on the gene transcription of several regulators of cellular growth and differentiation. CYP2E1 has been shown to break down retinoic acid and retinol, and its induction in response to ethanol intake has been postulated as the main reason for the depletion of vitamin A isoforms seen in chronic alcohol intake [133]. Depletion of retinoic acid has been observed to result in the upregulation of proliferative, anti-apoptotic transcriptional factors such as AP1. It is believed that disruption in retinoid metabolism may have a key role in carcinogenesis, even in the extrahepatic tissues [96].

As a by-product of the oxidation of ethanol by CYP2E1, a variety of reactive oxygen species are produced, leading to the development of a state of oxidative stress [69, 71, 96, 125, 128, 130]. In the past, investigations have determined that multiple polymorphisms of the gene that encodes CYP2E1 exist. One of these, the c2 allele for the RsaI/PstI polymorphism, was found to confer a significantly increased risk of developing oral cancer in Asian and mixed race subjects [134, 135]. This particular allele is associated with increased levels of gene transcription and an increased induction of the enzyme by ethanol consumption [136].

2.6.2.7 Oxidative Stress

Oxidative stress primarily refers to an excessive generation of reactive oxygen species within a cell. As mentioned above, induction of CYP2E1 as part of the MEOS leads to the production of reactive oxygen species. Examples of these agents include hydrogen peroxide (which may diffuse across lipid membranes), hydroxyl radicals (which are highly reactive), peroxynitrite (which may diffuse within cells) and superoxide [137]. Upon interaction with DNA, reactive oxygen species can cause multiple types of damage including base oxidation and fragmentation, single- and double-strand breaks, interstrand and intrastrand cross links and DNA–protein cross links [138]. Reactive oxygen species also produce a number of mutagenic DNA adducts, the most comprehensively studied of which is 8-hydroxy-2′-deoxyguanosine (8-oxo-dG). This lesion has been shown to induce significant errors during DNA replication and is considered mutagenic in humans [125, 137]. It has also been shown that levels of the adduct are significantly higher in cells isolated from the saliva of OSCC patients when compared to controls [139]. The various methods by which reactive oxygen species damage DNA have implications for carcinogenicity. For example, biopsies from patients with OSCC have been shown to demonstrate

greater amounts of reactive oxygen species and 8-oxo-dG adducts and reduced levels of antioxidant compounds [140, 141].

The importance of CYP2E1 in the development of oxidative stress is illustrated in the study by Bradford et al., who fed a high ethanol diet to normal mice and mice that were knocked out for functional CYP2E1. As a result, it was found that only the normal mice developed oxidative DNA adducts [142]. Mitochondrial DNA, which is susceptible to damage due to its poor repair capacity, may also be affected by reactive oxygen species. Changes in mitochondrial DNA have been found to be an important step in carcinogenesis. In addition to DNA, reactive oxygen species can also attack other cellular components such as proteins and lipids [124, 125, 128, 137, 138].

2.6.2.8 Lipid Peroxidation

Lipid peroxidation (LPO) is a common cellular process that occurs when the presence of intracellular oxidants (such as reactive oxygen species) leads to the oxidation of polyunsaturated fatty acid chains located within the phospholipid bilayer [137]. This causes a free radical reaction to occur, leading to the breakdown of the lipids and the formation of various by-products including aldehydes such as crotonaldehyde, acrolein, malondialdehyde and trans-4-hydroxy-2-nonenal (4-HNE) [143]. Whilst low levels of lipid peroxidation occur in physiological conditions, the process becomes significant during a state of excessive oxidative stress as excessive amounts of LPO by-products are formed [143]. These end products, many of which are reactive electrophiles, react with proteins and DNA and induce toxicity and mutagenesis [130, 144, 145]. In relation to these effects, high levels of LPO products have been found to be tightly associated with carcinogenesis in animal models [143]. In humans, LPO-related adducts of protein have been demonstrated to occur in the oral mucosa of patients with oral precancerous lesions and OSCC [128]. A recent study by Millonig et al. examined oesophageal biopsies from healthy patients and patients with upper aerodigestive tract cancer utilising immunostaining methods. As a result, they found a significantly increased number of LPO-related DNA adducts in the patients with cancer [130]. Consistent with the explained relationship between ethanol, CYP2E1 induction and the development of oxidative stress, it has been shown that chronic alcohol intake leads to the production of increased levels of LPO products [125, 128, 130]. In the Millonig et al. study mentioned previously, a strong correlation was found between ethanol intake, CYP2E1 staining and the prevalence of LPO-related DNA adducts [130].

Trans-4-hydroxy-2-nonenal (4-HNE) is one of the most abundantly produced aldehyde by-products of LPO. Intracellular levels of 4-HNE vary from 0.1 to 3 μM under physiological conditions. However, in times of oxidative stress, this level can vary from 10 μM to 5 mM. Due to its molecular structure, 4-HNE can readily react with both DNA and cellular proteins [146, 147]. These interactions can produce several adverse effects. Firstly, 4-HNE can directly react with DNA to create several types of DNA adducts. It may form the bulky exocyclic DNA adduct 6-(1-hydroxyhexanyl)-8-hydroxy-1,N-2-propano-2'-deoxyguanosine (4-HNE-dG) with guanosine, an adduct which has been observed to occur in both humans and

animals [146, 148]. Studies have shown that this adduct forms preferentially at sequences in the p53 gene (a tumour suppressor gene) and causes transversion mutations in human DNA [149]. Interestingly, mutations in the p53 gene are critical in oral carcinogenesis and have been reported in approximately 40 % of all OSCCs [150]. 4-HNE has also been shown to form an exocyclic variety of adducts termed 'ethenobases', including 1,N^2-ethenoguanine (εdG), 1,N^6-ethenodeoxyadenosine (εdA) and 3,N^4-ethenodeoxycytidine (εdC) [125, 137, 151]. Whilst εdG is the most prevalent adduct formed, εdA and εdC have been identified as highly mutagenic in mammalian cells, promoting base-pair substitutions [96, 125, 137, 152]. Secondly, the action of 4-HNE adducting to DNA repair enzymes directly interferes with the nucleotide excision repair system. In a study by Feng et al., it was shown that treatment of cells with a 100 μM solution of 4-HNE reduced base excision repair of benzo[α]pyrene-diol-epoxide (an exogenous carcinogen) damage by 50 % [143]. Thus, 4-HNE exhibits a mutagenic action via its interactions with cellular proteins and DNA. 4-HNE also has an important role in oral carcinogenesis; with a study by Warnakulasuriya et al., 4-HNE adducts were found in 80 % of dysplastic and malignant cells from oral biopsies [128]. The previously mentioned study by Millonig et al. also found that patients with upper aerodigestive tract tumours had a significantly greater number of εdA and εdC adducts than healthy patients [130].

Malondialdehyde (MDA) is another extensively studied aldehyde by-product of LPO. Whilst MDA is not as abundant as 4-HNE (it reaches concentrations of 20 μM in cells undergoing LPO), it is generally regarded as being the most mutagenic by-product of LPO [144, 153]. Similar to other aldehydes, MDA reacts with DNA to form several adducts, the most significant of which is pyrimido[1,2α]purin-10(3*H*)-one (M_1G) [144]. M_1G has been shown to be highly mutagenic in human cells, inducing base-pair substitutions and frameshift mutations [145, 153]. A study by Fink et al. found that M_1G induced a similar spectrum and frequency of mutations in *Escherichia coli* comparable to 1,N^2-PdG (an acetaldehyde adduct) [154]. In a similar manner to 4-HNE, MDA can also adduct to DNA repair enzymes, resulting in a reduced capacity for nucleotide excision repair. This was shown by Feng et al. in a 2006 follow-up to their previously mentioned 2004 study [153]. This is significant as the nucleotide excision repair system is responsible for the removal of the MDA-induced M_1G adduct. In this manner, MDA perpetuates its own mutagenicity [144]. MDA adducts to DNA, and proteins have been demonstrated to occur in oral epithelial cells [128, 155]. Consistent with the relationship between the induction of CYP2E1 and excessive LPO, Warnakulasuriya et al. found that increased levels of MDA adducts in oral epithelial cells had a strong correlation with increased levels of CYP2E1 staining [128]. Finally, increased levels of MDA have been found to be associated with cancers of the breast, gastric mucosa and cervix, and significantly higher levels of serum MDA were found in patients with oral cancer when compared to controls [145, 156]. A study by Sander et al. also found that significantly increased levels of MDA adducts were found in biopsies from patients with SCC of the skin compared to healthy patients [157].

2.6.2.9 Enhanced Penetration of Carcinogens

There is a growing body of evidence that suggests that ethanol may act to enhance the penetration of exogenous carcinogens across the oral mucosa. In 1976, Squier et al. showed that the incubation of porcine floor of the mouth mucosa with 5 % ethanol significantly enhanced its permeability to the carcinogen N-Nitrosonornicotine, found in tobacco smoke [158]. A similar study by Du et al. in 2000 found a similar effect, except the greatest increase in penetration occurred at a concentration of 25–30 % ethanol [159]. In both studies, a higher concentration of 50 % ethanol did not significantly increase permeability of the oral mucosa. It was suggested that local concentrations of ethanol this high instead have a fixative effect on the tissue instead of a permeating effect [158, 159]. One of the methodological flaws in these two studies is that the samples were exposed in ethanol for up to 24 h [158, 159]. This was accounted for in a 2001 study by Howie et al., who reduced the exposure time to 1 h and still found that penetration of the high molecular weight molecule albumin was significantly enhanced at a concentration of 15 % ethanol in human oral mucosa [160]. Whilst other tissues have been tested, this phenomenon is most marked in the mucosa of the floor of the mouth [158, 159]. Interestingly, the floor of the mouth is regarded as a high-risk site with regard to OSCC, suggesting a possible relationship [161]. Ethanol is a well-known penetration enhancer in skin, being used in a variety of transdermal delivery systems. It has been shown to achieve this effect by removal of barrier lipid from the stratum corneum [162]. Ganem-Quinitar et al. found a similar loss of barrier lipid occurring in porcine palatal mucosa exposed to ethanol. Interestingly, the lipid types that were the most reduced were those found predominately in non-keratinised mucosa such as the floor of the mouth [163]. On the other hand, Howie et al. found that lipid fractions within human oral mucosa were unchanged following exposure to ethanol. As a result, they suggested that ethanol may disrupt lipid architecture, thus opening up a route for the penetration of carcinogens [160]. Chronic ethanol administration to rats was also found to increase the penetration of N-Nitrosonornicotine across the oral mucosa, suggesting that there may also be a permeating effect from either chronic local or systemic exposure in addition to the noted acute local effects [164].

2.6.3 Alcohol-Containing Mouthwashes

Ethanol is a key ingredient in a majority of commercially available mouthwashes, acting as a solvent, preservative, antiseptic and caustic agent. The concentration that occurs varies between products, but it can be as high as 26 % v/v. As this concentration exceeds that found in certain types of alcoholic beverages, alcohol-containing mouthwashes have come under scrutiny regarding any causative link to OSCC. There have been a number of epidemiological studies and reviews examining this relationship. In addition to this, a number of in vivo and in vitro studies have examined the local effects of alcohol-containing mouthwashes in the oral cavity.

2.6.3.1 Alcohol-Containing Mouthwashes and Oral Cancer: Epidemiological Evidence

The possibility of a relationship between mouthwash use and OSCC was first raised following a case–series and case–control study in 1979 [165]. Including this publication, there have been 19 case–control studies that have examined mouthwash use in patients with OSCC [111, 165–182]. There also exist a number of reviews which address the same issue [162, 174, 183–189]. Whilst this represents a broad base of evidence, the possible carcinogenicity of alcohol-containing mouthwashes is still a controversial issue. Overall, the case–control studies provide conflicting results regarding the excess risk of OSCC, if any, afforded by the use of mouthwashes. Several have reported a significant increase in risk, whilst others have reported an insignificant increase in risk, no change in risk or even a reduced risk [111, 165–182], 190]. Similarly, the reviews are divided in their support for and against a relationship [174, 183–189].

One of the reasons for these conflicting results is the great variation in study design between the primary case–control studies. In the 18 studies that exist, there is considerable difference with regard to the information relating to mouthwash use that was requested from study participants. The main issue is that only six studies [111, 170, 173, 174, 180, 181] specified when patients were using alcohol-containing mouthwashes, whereas the rest merely investigated the association between 'mouthwash use' and OSCC [111, 170, 173, 174, 180, 181]. As ethanol has been demonstrated as a carcinogen related to OSCC development, it is obviously important that it be specified that exposure to alcohol-containing mouthwash, rather than just mouthwash, is being assessed. Reporting on other variables relating to mouthwash use was also sporadic, with 15 assessing the frequency of use, 8 assessing the history of use, 3 assessing the retention time in the mouth and 3 assessing the reasons for mouthwash use [111, 165–182, 190]. Studies also varied regarding the site of cancer, with most restricting the case definition to SCC of the oral cavity and pharynx, whilst others also included laryngeal sites. This amount of heterogeneity with regard to study design means that it is difficult to compare the individual results of each study [111, 165–182, 190].

Another difficulty regarding the quantification of the effect (or not) of alcohol-containing mouthwashes on OSCC risk is the high incidence of their use in patients who also smoke and/or drink alcohol [174]. As mentioned previously, both smoking and alcohol ingestion are independent risk factors in the development of OSCC. It is theorised that the high level of overlap between these habits and alcohol-containing mouthwash use may lead to an overestimation of the risk imparted by mouthwash use. An example of this is seen in the study by Kabat et al., one of the few to assess reasons for patient mouthwash use. It was found that female subjects were significantly more likely to use mouthwash to hide the odours of tobacco (OR = 3.3, 95 % CI 1.24–8.75) and alcohol (OR = 3.25, 95 % CI 1.03–10.3) than food odours (OR = 0.66, 95 % CI 0.3–1.43) or dental infections (OR = 0.72, 95 % CI 0.27–1.94) [169]. It has also been theorised that underreporting of smoking or alcohol usage amongst cases may lead to the overestimation of the effect of alcohol-containing mouthwash usage [191]. However, it has rightly been pointed out that

similar underreporting amongst controls would lead to a converse underestimation of risk [192]. The effect of these confounding factors can be seen in a 2013 study by Eliot et al., who found that ≥1/daily mouthwash use compared to non-users showed a slightly increased risk of developing OSCC in heavier drinkers (>2 drinks/day) (OR = 1.14, 95 % CI 0.99–1.32) compared to non-drinkers (OR = 1.08, 95 % CI 0.87–1.34) and ever smokers (OR = 1.17, 95 % CI 1.01–1.23) compared to never smokers (OR = 1.10, 95 % CI 0.96–1.25) [180]. In this case, the question remains whether the increased risk associated with combined smoking/drinking and mouthwash use compared to mouthwash use alone is indicative of a cumulative or synergistic effect on risk or due to the presence of smoking and alcohol consumption as already established risk factors for the development of OSCC.

In a case–control study in 1983, Wynder et al. identified that the subgroup of non-smoking, non-alcohol consuming women who used mouthwash daily had an increased risk of developing OSCC (OR = 3.63, 95 % CI 1.48–8.92) [167]. It has been suggested that due to the absence of classical risk factors, the non-smoking, non-drinking demographic would be the most likely to demonstrate a carcinogenic action of alcohol-containing mouthwash [173]. A follow-up study limited to women did show a non-significant elevated risk in non-smokers/non-drinkers who used mouthwash (OR = 1.38, 95 % CI 0.42–4.55). However, this was hampered by the small population of the subgroup (8 cases, 7 controls) [169]. Winn et al. in 2001 noted a similar non-significant risk increase in the same subgroup (OR = 2.8, 95 % CI 0.8–9.9), as did Divaris et al. [173, 178]. On the other hand, Winn et al. in 1991 found that odds ratios for non-smoking, non-drinking males and females using mouthwash were actually less than those of the general study population [170].

Another important aspect that has an impact on the epidemiological evidence related to mouthwash use is the prevalence of industry sponsorship. Two of the reviews mentioned above, the Gandini et al. meta-analysis, the Shapiro et al. article on the statistical effects of underreporting of alcohol and tobacco usage and the Cole et al. review and reanalysis of the Winn et al. 1991 dataset, all declare some form of industry affiliation [174, 183, 184, 191, 193]. This comes primarily from the pharmaceutical companies Warner and Lambert, Pfizer and Johnson & Johnson, who have all held ownership of the mouthwash brand ‘Listerine’. The unaffiliated review by Lachenmeier et al. noted that the industry-supported studies had much more positive conclusions (i.e. no relationship between alcohol-containing mouthwashes and OSCC) than other independent reviews and indicated that there may be some form of bias occurring [162].

In 2012, Gandini et al. published a meta-analysis of all known epidemiological studies examining the relationship between mouthwash use and oral cancer (totalling 4,484 cases and 8,781 controls). After analysis, it was determined that there was no significant association between mouthwash use and oral cancer (RR = 1.13; 95 % CI 0.95–1.35), no significant risk associated with daily use (p = 0.11) and no significant association when it was specified that mouthwashes contained alcohol (RR = 1.0; 95 % CI 0.39, 2.60) [193]. However, it has been questioned whether a meta-analysis with even this number of subjects would have enough statistical

power to detect a low but significant risk imparted by regular use of alcohol-containing mouthwashes [194].

Overall, the heterogeneity in design and results between epidemiological studies and reviews makes it impossible to accurately judge the relationship between use of alcohol-containing mouthwashes and the development of OSCC. Further consistently designed studies with large numbers of participants, stringent examination of all the variables related to mouthwash use, specification of ethanol content in mouthwash and detailed control for alcohol and tobacco consumption are required before a definitive relationship can be established or discredited.

2.6.3.2 Alcohol-Containing Mouthwashes and Oral Cancer: Mechanistic Evidence

In addition to the epidemiological studies mentioned above, there also exist a number of in vitro and in vivo studies that investigate the effects of alcohol-containing mouthwashes on human cells and in the oral cavity. Whilst consistent epidemiological findings are necessary to establish a causal relationship between alcohol-containing mouthwash use and the development of OSCC, these studies may provide an insight regarding the local effects and possible carcinogenic mechanisms.

As mentioned previously, the production of acetaldehyde from ethanol is regarded by the International Agency for Research on Cancer as a carcinogenic process [67]. It has also been outlined that the metabolism of ethanol to acetaldehyde can occur in the mouth due to the presence of ADH in human oral epithelial cells and ADHs produced by commensal bacteria. Lachenmeier et al. conducted a trial in healthy human volunteers to quantify the amount of acetaldehyde produced in the oral cavity following 30 s of exposure to 13 different alcohol-containing mouthwashes whose alcoholic concentration varied from 6.8 % v/v to 26.8 % v/v [195]. It was found that whilst no acetaldehyde was detectable prior to exposure, an average concentration of 52 ± 14 μM (range 11–105 μM) acetaldehyde could be detected in the saliva at 2 min post-exposure. This value had been reduced to 15 ± 7 μM (range 0–37 μM) at 10 min post-exposure. As is evidenced by the range of values, a large amount of interindividual variation was present [195]. The authors noted that these were significant findings as it has been proven that formation of the highly mutagenic 1,N^2-PdG adducts can occur at acetaldehyde concentrations as low as 100 μM and sister chromatid exchanges can occur in mammalian cells at concentrations as low as 88 μM [114, 121]. Added to this is the recent evaluation by Salaspuro et al. that leads to the conclusion that the mutagenic threshold of acetaldehyde in saliva falls between 50 and 150 μM [196]. A similar study utilising alcohol-containing mouthwashes and human volunteers found significantly raised salivary acetaldehyde concentrations ranging from 43.8 to 97.0 μM at 1 min post-exposure [197]. However, as mentioned previously, Homann et al. found that the administration of chlorhexidine mouth rinse significantly reduced local acetaldehyde production from ethanol due to its action of reducing oral microbe levels [104]. This was also demonstrated in a separate trial in which human volunteers rinsed with an alcohol-containing mouthwash for 30 s followed by measuring of

salivary acetaldehyde at several points. At 2 min post-exposure, the essential oil mouthwash resulted in an average salivary acetaldehyde concentration of 44.3 μM (range 35.2–63.6 μM) compared to an equivalent solution of ethanol which resulted in a concentration of 72.6 μM (range 46.5–111.2 μM) [198]. Whilst this is a significantly lower concentration, the alcohol-containing mouthwash group still demonstrates individual values that are within Salaspuro's theoretical concentration range of mutagenicity. It should also be noted that this study received funding from Johnson & Johnson, the manufacturers of Listerine. Overall, it would appear that whilst the antibacterial properties of alcohol-containing mouthwashes reduce the level of acetaldehyde production in the oral cavity (compared to an equivalent solution of ethanol), the constituent ethanol of the mouthwash still results in a production of acetaldehyde to the level where mutagenic effects may occur.

Another human in vivo study was conducted by Zamora-Perez et al., who investigated the incidence of nuclear abnormalities in exfoliated buccal cells from three groups of participants: one group who had used an alcohol-containing mouthwash twice a day for 30 days, another group who had used an alcohol-free mouthwash and a group who had used neither. It was found that compared to the two alcohol-free groups, use of the alcohol-containing mouthwash resulted in significantly higher numbers of nuclear abnormalities such as micronucleus, binucleated cells and nuclear budding. This is a significant finding given that nuclear abnormalities are strong markers of genotoxicity [199].

Several other in vitro studies related to alcohol-containing mouthwashes have been conducted. Rodrigues et al. investigated the ability of three different mouthwashes to induce genetic mutations using the *Drosophila melanogaster* somatic mutation and recombination test [200]. This test is recognised as being useful in evaluating the genotoxicity of environmental agents in humans [201]. Rodrigues et al. found that the test mouthwash with the highest percentage of ethanol (16.8 %) induced a significant number of mitotic recombinations. Further investigation determined that it was the ethanol present in the mouthwash, not the active ingredient (cetylpyridinium chloride), that was causing the genotoxicity. The authors theorised that acetaldehyde was the causative agent in this genetic damage [200]. Another study used the single cell gel electrophoresis assay to measure the induction of DNA strand breaks in cultured human oral epithelial cells following exposure to a dilute solution of an alcohol-containing mouthwash. Significantly greater DNA damage was observed when compared to an ethanol-free control group [197].

In vivo studies in animals investigating the effects on the oral mucosa of long-term topical exposure to alcohol-containing mouthwashes are non-existent. However, several animal studies that utilise pure ethanol in similar concentrations to commercially available mouthwashes do exist. Maier et al. investigated the changes that occurred in the oral mucosa of rats that were fed a diet containing 6.6 % v/v ethanol for 6 months. It was found that the floor of the mouth, lateral tongue and ventral tongue epithelium of ethanol-exposed rats had significantly enlarged basal cell nuclei, basal cell hyperplasia, altered epithelial stratification and a greater percentage of cells in the S phase of the cell cycle. It was also found that the mean thickness of the floor of the mouth mucosa was significantly reduced in

ethanol-exposed rats. The authors concluded that chronic topical exposure to ethanol induced both oral mucosal atrophy and hyper-regeneration, and this likely resulted in an increased susceptibility to carcinogens [202]. Another study in rats conducted by Simanowski et al. utilising 6.4 % v/v ethanol over 5 months found that the proliferation rate of oesophageal epithelial cells was significantly increased in rats exposed to ethanol. This is a significant finding given that mucosal hyperproliferation is an established risk factor in other malignancies such as colorectal carcinoma [203]. Muller et al. conducted a similar study in rabbits, except utilising higher concentrations of ethanol (20 % v/v, 40 % v/v and 96 % v/v) over 12 months. The oral epithelium of ethanol-exposed rabbits developed abnormalities such as dyskeratosis, surface keratosis, increased basal layer density and an increased number of mitotic figures [204]. Given that several problems exist in relating the results of these studies to alcohol-containing mouthwash use (increased number of exposures, length of exposure, consumption of ethanol, etc.), further animal studies are required to investigate the local effects of long-term topical exposure to alcohol-containing mouthwashes on the oral mucosa.

Overall, mechanistic evidence from these in vivo and in vitro studies suggests that the metabolism of the ethanol in alcohol-containing mouthwashes can produce a significant amount of acetaldehyde in the oral cavity (even up to a level where genetic damage may occur). The occurrence of genetic damage after exposure to alcohol-containing mouthwashes has been demonstrated in animal models. It is also worth noting the remainder of the in vitro studies investigated the acute toxic effects of short-term exposure to alcohol-containing mouthwashes, not chronic effects related to repeated exposures [205–208]. However, the production of significant epithelial abnormalities in the oral mucosa of animals via chronic topical ethanol exposure has been characterised in a number of studies. Greater investigation is required to characterise the events and pathways by which genetic damage may occur through chronic exposure to alcohol-containing mouthwashes.

It is worth noting however that Lachenmeier et al. (in their review) questioned the overarching need for the addition of ethanol to the formulation of antibacterial mouthwashes [162]. They noted that multiple studies have shown that alcohol-free mouthwashes are just as effective and have been shown to have a lower incidence of adverse effects than their ethanol containing contemporaries [209–216]. An interesting point was demonstrated in a study that investigated the production of salivary acetaldehyde after rinsing with either an alcohol-containing mouthwash or an alcohol-free variant. It was found that use of the alcohol-containing mouthwash resulted in a significantly greater peak in salivary acetaldehyde than the alcohol-free version. As the study was run over 2 weeks with the same subjects, it was also found that even daily use of an alcohol-containing mouthwash induced almost fivefold greater amounts of salivary acetaldehyde than an alcohol-free counterpart [217].

2.6.3.3 Unifying Hypothesis

The underlying arguments for and against an increased risk of oral cancer with chronic mouthwash use have recently been explored in an excellent review by Currie and Farah, where they propose a unifying hypothesis for pathways involved

in oral mucosal carcinogenesis following the use of alcohol-containing mouthwash taking into account epidemiological and mechanistic evidence [218]. Within this hypothetical model, and within the overall framework of field cancerisation related to environmental risk factors such as tobacco, ethanol and betel quid, regular topical exposure to alcohol-containing mouthwash could theoretically have several effects from a carcinogenic viewpoint.

A brief exposure has already been shown to induce a sharp rise in the level of salivary acetaldehyde to a point where there is the potential for mutagenic events to occur [195, 196, 219]. As noted, the antibacterial action of alcohol-containing mouthwashes does reduce the contribution to salivary acetaldehyde by oral flora; however, the use of an alcohol-containing mouthwash generates significantly higher levels of salivary acetaldehyde compared to a non-alcoholic mouthwash, even after 2 weeks of twice-daily use, after which the oral flora would be thoroughly suppressed [217]. This demonstrates that even in the relative absence of contributing bacteria, ethanol in mouthwashes drives increased salivary acetaldehyde. In addition to the direct generation of a carcinogen, ethanol also has indirect effects such as increased mucosal permeation and induction of cytochrome P450 2E1, which act to enhance the actions of tobacco-related carcinogens evidenced by a greater than multiplicative increase in OSCC risk associated with concurrent smoking and drinking [38, 96]. This is likely to be relevant to alcohol-containing mouthwash use, as mouthwash users who smoke are at greater risk of developing OSCC than non-smoking users [170, 176]. These combined effects may result in continued mutagenic events within an already sensitised field, promoting continued epithelial transformation. The effects of alcoholic beverages in this respect have already been seen, as it has recently been shown that continued consumption of alcoholic beverages after the development of OSCC significantly increases a patient's risk of developing a second primary OSCC presumably from continued transformation within the sensitised field [220].

It is possible to identify several groups of alcohol-containing mouthwash users who could theoretically be at higher risk with chronic use of high alcohol-containing mouthwash. Firstly, subjects who smoke and use alcohol-containing mouthwash are regularly exposed to both tobacco carcinogens and ethanol, the synergy of which has been highlighted above. Epidemiological studies have also shown that current and past smokers are more likely to use mouthwash [166, 167, 169, 170, 180]. Secondly, use of alcohol-containing mouthwash by patients with oral epithelial dysplasia has the potential for concern, as continued exposure to ethanol may act to facilitate progression towards malignancy. The presence of dysplastic lesions in the oral cavity presents areas of the field that are arguably more sensitive to malignant transformation in response to environmental stimuli, given the dysregulation of cellular processes that already exists within these cells. Patients with oral epithelial dysplasia tend to be smokers, and the oral epithelium in these patients is already transformed, placing them at heightened risk of further cellular and molecular damage should they engage in chronic use of alcohol-containing mouthwash. It is also possible that the discovery of an oral lesion by a patient may act as the motivating factor for mouthwash use, which would place the patient at increased risk of further damage to an existent lesion. A handful of studies have investigated the relationship

between mouthwash use and the development of oral epithelial dysplasia. Morse et al. in a case–control study (127 cases, 127 controls) found no evidence for a relationship, even when data was stratified by frequency of use, history of use, retention time and alcohol content [221]. On the other hand, Dost et al. found a higher proportion of dysplastic lesions in users of mouthwash; however, this increase did not approach statistical significance [222].

Whilst there is still controversy regarding the possible effects imparted by alcohol-containing mouthwash use, in the meantime, it is reasonable for clinicians to take steps to mitigate against any possible risk. As mentioned previously, ethanol is the ingredient in mouthwashes that has led to increased scrutiny, and in response to this, a number of ethanol-free antibacterial mouthwashes have become available on the general market, particularly in recent years following renewed calls for cessation of their regular use. Studies have shown that these formulations are as effective and have been shown to have a lower incidence of adverse effects than their ethanol-containing counterparts [209–213, 215, 216].

2.6.4 Microorganisms

The role of microorganisms in the aetiology of cancer is gaining interest. A recent study investigating the global burden of cancers attributable to infections showed that out of the 12.7 million new cancer cases that occurred in 2008, 1.9 million new cancer cases were attributable to infections by *Helicobacter pylori* bacteria, hepatitis B and C viruses and human papillomaviruses, mainly gastric, liver and cervix uteri cancers, respectively. Around 30 % of infection-attributable cases were in people younger than 50 years [223].

Poor oral hygiene may be an independent risk factor in oral cancer, and this has been supported by findings that head and neck cancers are more frequent in people with poor oral hygiene [176, 224]. Poor dental status, tooth loss and periodontal disease have been shown to increase the risk of oral cancer [225, 226]. Further, clear differences have been observed when comparing the microbial population on oral mucosa between healthy and malignant tissues [227]. The mechanism by which bacterial infections may cause cancer is not clear. Bacterial infections may induce cancer by triggering cell proliferation, inhibiting apoptosis, interfering with cellular signalling pathways and up-regulating tumour promoters [228]. The poly-microbial burden caused by oral biofilms can have a mutagenic interaction with saliva which may act as cofactors in the carcinogenic process [229]. Another possible bacterial carcinogenic mechanism is the oral production of the highly carcinogenic acetaldehyde from alcohol or tobacco by several oral microorganisms. This capacity of oral bacteria may explain the significant link between poor oral hygiene and oral cancer in heavy smokers or drinkers [230, 231].

2.6.4.1 Human Papillomavirus

The human papillomavirus, a DNA virus, causes the vast majority of cervical, but also a substantial proportion of other anogenital and head and neck cancers and

specific types have been linked to certain cutaneous cancers [232]. More than 130 types have been identified in this heterogeneous virus family. These types have been classified into low- or high-risk groups according to their potential for oncogenesis based on persistent infection [233]. There is evidence that genotypes 16 and 18 play a role in the aetiology of head and neck cancers, particularly tonsil and oropharyngeal carcinoma [234]. A recent meta-analysis found that HPV16 accounted for 82.2 % (95 % CI 77.7–86.4) of all HPV DNA-positive head and neck SCC cases. The carcinogenic mechanism of viral infections is believed to be mainly related to their interaction with gene regulation [235]. The virus is present in oropharyngeal SCC either in integrated or episomal form [236].

There has been an overall increase in the incidence of base of tongue cancer with an increase in the prevalence of HPV in these tongue cancers [232, 237]. Prevalence of HPV DNA is estimated to be 45.8 % (95 % CI 38.9–52.9) for the oropharynx and 24.2 % (18.7–30.2) for the oral cavity [233, 238]. Lingen however reported the aetiological fraction for high-risk HPV in oral cavity SCC as 5.9 %, with significant attribution to HPV16 at 3.7 % (95 % CI 1.8–5.5), whilst other risk HPV types were attributed as 2.2 % (95 % CI 0.8–3.6) [238–240].

Current techniques for the clinical diagnosis of HPV-associated HNSCC include the detection of HPV DNA, RNA and the HPV surrogate marker, p16 in tumour tissues as well as HPV-specific antibodies in serum [241, 242]. The HPV protein E7 degrades the retinoblastoma protein leading to aberrant overexpression of p16, thus its use as a surrogate marker [242]. Importantly, in both head and neck SCC and specifically oropharyngeal SCC, HPV-positive patients have a significantly lower disease-specific mortality and are less likely to experience progression or recurrence of their cancer compared to HPV-negative patients [238, 240]. Prophylactic vaccines targeting HPV16 and HPV18 have the potential to prevent a substantial fraction of head and neck squamous cell carcinomas worldwide, in particular oropharyngeal cancers, in both males and females [233, 238].

2.6.4.2 Candida

There has been increasing clinical and experimental evidence to suggest a putative role for yeast *Candida* in the multistep process of oral mucosal carcinogenesis. *Candida* can cause a spectrum of oral mucosal lesions. In particular, chronic hyperplastic candidosis, also known as candidal leukoplakia, has been postulated that this variant of oral candidosis carries a significant risk of malignant transformation [243, 244].

A considerable number of clinical and experimental investigations demonstrated that yeast and bacteria can promote carcinogenesis either directly or indirectly [245] and that some *C. albicans* biotypes may contribute more to carcinogenesis than others [246].

A statistically significant association has been shown between fungal colonisation and both malignant and dysplastic sites [227, 247]. Further, there is correlation of increasing degree of epithelial dysplasia with yeast carriage [248]. The majority of nonhomogenous leukoplakia, associated with a higher malignant transformation

rate than homogenous leukoplakias [249, 250], are invaded by yeast, particularly *Candida albicans* [251–253]. Most recently, data from a matched case–control study investigating the relative risk of oral *Candida* colonisation amongst other traditional risk factors of oral cancer showed that both oral *Candida* carriage (OR = 3.242; 95 % CI 1.505–6.984) and high level of colonisation (OR = 3.587; 95 % CI 1.153–11.162) were significant risk factors in oral cancer in addition to regular daily alcohol consumption (OR = 4.253; 95 % CI 1.351–13.386) [254]. More importantly, the risk effect was highly additive, but less than multiplicative when *Candida* presence was conjugated with alcohol consumption (OR for *Candida* presence *plus* current/daily alcohol drinking = 9.288; 95 % CI 2.022–42.6), suggesting fungal alcohol metabolism being a probable link [254]. It has been suggested that the presence of *Candida* in association with dysplastic or malignant lesions may represent a secondary infection with a pre-existing altered epithelium [251]; however, clinical resolution of chronic hyperplastic candidal lesions and the reduction in extent of dysplasia after antifungal therapy have emphasised a direct aetiological role [255].

Several mechanisms by which *Candida* may promote oral cancer have been proposed. *Candida* infection might disturb epithelial activity and predispose to neoplastic change by playing a promoter role in itself or by complementing known promoters of carcinogenesis [256–259]. One such mechanism is the ability of yeast isolates, in particular *C. albicans,* to form N-nitrosobenzylmethylamine [260–262]. This carcinogen may directly or indirectly, or in concert with other carcinogens, activate specific proto-oncogenes and initiate the development of malignant lesions [263]. Another mechanism is activation of procarcinogenic compounds into carcinogenic forms, such as acetaldehyde from ethanol, by yeast and other oral flora [110, 263–266]. It is still unclear if chronic microbial-induced inflammation may influence mucosal carcinogenesis by up-regulation of pro-inflammatory cytokines and growth factors [228, 267]. Although inflammatory mediators, including cytokines, offer protection by destroying invading pathogens, they can inhibit apoptosis and enhance cell proliferation, both of which may be involved in promoting mutation and carcinogenesis [268, 269]. Thus, over a considerable time, data has accumulated that suggests *C. albicans* may promote OSCC development, but the exact role of this yeast in neoplastic change is yet to be fully elucidated.

2.6.5 Diet

It has been estimated that dietary factors may account for approximately 20–30 % of all cancers in Western and developing countries [1, 270]. Poor diet is a significant risk factor for all cancers, including oral cancer [224]. This concept is based on case–control and cohort studies and from animal and experimental studies. High fruit and/or vegetable intake has been shown to decrease the risk of oral cancer [271]. In a recent review of the effects of lifestyle on oral cancer risk worldwide, Petti has shown that 10–15 % of oral cancer is attributable to

micronutrient deficiency [68]. A detrimental lifestyle was defined as one high in fat and/or sugar intake, leading to low fruit and/or vegetable consumption [68]. In another study [270], the relation between single aspects of diet and the risk of oral and pharyngeal cancer has been reviewed from six cohort studies and approximately 40 case–control studies. In this study, fruit and vegetable intake were inversely related to risk. High vegetable consumption had a pooled relative risk (RR) of 0.65 from four cohort studies on upper aerodigestive tract cancer and 0.52 from 18 case–control studies on oral and pharyngeal cancer, corresponding to RR of 0.78 and 0.55, respectively, for high fruit intake in these studies [270]. A protective effect was noted for micronutrients such as beta-carotene, vitamin C and flavonoid, though it was difficult to disentangle their potential effect from that of fruit and vegetable. Whole grain, but not refined grain, intake has been favourably related to cancer risk, whilst inconsistent results have been noted for beverages and other nutrients such as meat, milk and dairy products. Fish has also been suggested to reduce oral and pharyngeal cancer risk, possibly on account of its high content of *n*-3 fatty acids, which have been shown to have a chemopreventive role on various neoplasms [270].

Similar effects have been demonstrated for oral potentially malignant disorders. In a population-based case–control study in Japan comprising 48 cases of oral leukoplakia and 192 control subjects, logistic regression analysis showed that high serum levels of beta-carotene were significantly associated with low risk of oral leukoplakia with an odds ratio of 0.16 [272]. Intervention studies are also promising in this regard. In India, data from a major double-blind placebo-controlled study displayed that up to one-third of subjects showed regression of oral leukoplakias after 12 months of supplementation with β-carotene [273]. It is believed that the maximum protective effect of plant food may be achieved by the combination of different nutrients and that the more frequent consumption of fruits and vegetables is a non-specific indicator of a more affluent and better-planned diet [270, 274].

The beneficial effect of fruit and vegetable has been attributed to several micronutrients such as flavonoid, polyphenol and fibres [270]. It seems that these components display both complementary and overlapping mechanisms of action, including antioxidant effects, maintenance of immune function, binding and dilution of carcinogens in the digestive tract, thus reducing their toxicity, maintaining appropriate cell differentiation and inhibiting cell proliferation [45, 275–277].

Micronutrients such as vitamin A and related carotenoids (in particular beta-carotene), vitamins C and E, and selenium are thought to be protective against most epithelial cancers, and much of the effect is due to their antioxidant activities [1]. The anticarcinogenic effect of antioxidants is achieved by reducing free radical reactions that can cause DNA damage and changes in lipid peroxidation of cellular membranes [278]. Recently, there has been an increasing interest in foodstuffs and drinks containing antioxidants, such as green tea, which contains high levels of polyphenols, a powerful antioxidant able to counteract both initiation and promotion of carcinogenesis [1, 224].

2.7 Immune Deficiency and Transplantation

Several studies have shown that patients with human immunodeficiency virus (HIV) are at higher risk for certain types of cancer including lip, oral cavity, pharyngeal and oesophageal cancer compared to the general population [279–281]. This is more often the case for cancers that are related or suspected to be related to a known infectious cause [279]. Patients with acquired immunodeficiency syndrome (AIDS) are at higher risk for three malignancies, commonly referred to as AIDS-defining cancer: Kaposi sarcoma, non-Hodgkin lymphoma and cervical cancer [281]. Although classified as a non-AIDS-defining cancer, a meta-analysis showed cancers of the oral cavity and pharynx to have an increased incidence in people with HIV/AIDS as well as transplant recipients [282].

Solid organ transplant recipients who receive iatrogenic immune suppression are two- to fourfold more susceptible to developing cancer [283, 284], this finding being generally attributed to immunosuppression. The cancer spectrum of solid organ transplant recipients is similar to that of patients with HIV [279], and as expected, malignancies caused or attributed to viral infections such as non-Hodgkin lymphoma and Hodgkin lymphoma (Epstein-Barr virus [EBV]), Kaposi sarcoma (human herpesvirus 8) and liver cancer (hepatitis C and B viruses) are the most frequently seen in these patients [282]. Engles et al. matched the US solid organ transplant registry (inclusive of more than 175,000 transplant recipients) with state and regional cancer registries and found non-Hodgkin lymphoma and lung cancer to have the highest incidence between infection-related and non-infection-related malignancies, respectively [282]. In this study, a significant increase in the number of oropharyngeal cancer (2.01 fold), lip cancer (16.78 fold) and other oral cavity and pharyngeal cancer (2.56 fold) was reported in solid organ recipients [282]. A deficient immune system, the minor role of HPV in carcinogenesis of oral cancer and the fact that immunodeficient/immunosuppressed patients are more susceptible to infectious-related cancers explain the higher rate of OSCC in these groups of individuals.

2.8 Genetics, Syndromes and DNA Repair Defects

OSCC is known to be related to environmental causes with a long history of alcohol and/or tobacco abuse as the most important risk factors. However, a number of other aetiological factors including diet, viral infection, immunodeficiency, socioeconomic status and genetic predisposition have been suggested to increase an individual's risk for developing OSCC [285–287]. Environmental contributors to carcinogenesis of OSCC exert their impact through modifying genetic material of the cells, leading to (a) self-sufficiency in growth and (b) possession of unlimited replicative potential and non-response to either (c) anti-growth or (d) apoptosis signals. In addition cancer cells are capable of (e) metastasis, (f) have sustained angiogenesis, (g) have a deregulated cellular metabolism and (h) avoid immune destruction [288–294]. Despite the building body of evidence detailing these changes, the exact nature and sequence of these alterations are yet to be elucidated.

In addition to genetic changes induced by environmental factors, hereditary syndromes and genetic predisposition due to deficient DNA damage repair mechanisms have also been proposed to initiate and promote malignant transformation of epithelial cells. DNA damage repair could be defined as a range of cellular and molecular responses with the aim of restoring the normal DNA sequence which could be damaged due to either endogenous causes such as reactive oxygen species from normal metabolic by-products or exogenous agents such as ultraviolet light. DNA damage occurs at a rate of 1,000 to 1,000,000 molecular lesions per cell per day [295]; therefore, in the absence of effective repair mechanisms, genetic instability and neoplasm formation would be inevitable.

One of the most widely studied hereditary deficiencies of the DNA damage repair mechanisms is hereditary non-polyposis colon cancer, otherwise known as Lynch syndrome which is due to inherited mutations in DNA mismatch repair (MMR) pathway. Mismatch repair (MMR) is a strand-specific, post-replicative DNA repair mechanism which plays a key role in sustaining genomic integrity by repairing DNA biosynthetic errors, single-base substitution mismatches and insertions/deletions in microsatellites [296]. Human MutS homologues 2 and 6 (hMSH2 and hMSH6, respectively) are key components of MMR whose role in recognition of DNA damage is widely acknowledged, and human MutL homologue1 (hMLH1) and human postmitotic segregation 2 (hPMS2) facilitate MutS homologues function and recruit additional proteins to complete the remaining stages of DNA repair [297]. Expression of hMLH1, hMSH2 and hPMS2 has been shown to be lost or significantly reduced in HNSCC and lesions with a high degree of dysplasia [298–306]. Allelic imbalance in hMLH1 has been suggested to be an aetiological factor in head and neck carcinogenesis [304], and promoter methylation of this gene has been shown to be an early event in oral carcinogenesis [298].

Another potentially lethal form of DNA damage is double-strand break (DSB); if left unrepaired, these lesions can cause genomic instability, chromosomal translocation and cell death. Deficiency in Fanconi anaemia (FA) family of genes in addition to breast cancer (BRCA) pathways which mainly repair DSB lesions can result in malignant transformation [307]. Although life-threatening results of deficiencies in BRCA-related pathways mostly include an increased (up to 80 %) life time risk for breast and ovarian cancer [308], there is overwhelming evidence pointing to higher susceptibility of patients with mutations in Fanconi anaemia family of genes and BRCA1/BRCA2 to other types of cancer including HNSCC and OSCC [309–313].

Deficiency of other DNA damage repair proteins including O^6-methyl-guanine DNA methyltransferase (MGMT) [314], X-ray repair cross-complementing group 1 (XRCC1) [311, 315] and human 8-oxoguanine DNA N-glycosylase 1 (hOGG1) [316] have also been associated with OPML and OSCC.

Although predictors of transformation or progression of OPML to OSCC or in general carcinogenesis of OSCC have been the main focus of numerous studies, determining the exact nature of genetic contributors to OSCC development is complicated by the heterogeneous nature of this tumour. In general, carcinogenesis of OSCC is believed to be due to sequential mutations that may result from genetic instability, either inherited or acquired over time.

2.9 UV and Lip Cancer

Most cancers of the lip are squamous cell carcinomas, and these represent 25–30 % of all carcinomas of the oral cavity [317]. Up to 90 % of the lip tumours have been classified as lower lip SCC [317, 318]. Although SCC has been frequently reported to be the most common malignancy of both upper and lower lips [319], some studies have found a larger proportion of basal cell carcinomas (BCCs) on the upper lip [320].

Due to its anatomical location, it is reasonable to assume lip cancers share some risk factors with oral cavity and skin tumours. Fair-skinned males over 50 years of age are considered a high-risk population [318, 321]. Cumulative life time exposure to ultraviolet (UV) light however remains to be the most important risk factor for developing lip cancer [322], with smoking, alcohol consumption and low socioeconomic status acting as confounding risk factors [319]. Although UV radiation is an important risk factor for the development of both SCC and BCC, cumulative life time exposure to UV seems to have a stronger relationship with SCC, whilst intermittent sun exposure especially during childhood has been proposed to be associated with BCC [323].

UV radiation creates keratinocytes with potential for malignant transformation either through direct damage to cellular nucleic acids (UVB) or formation of reactive oxygen species (UVA) [323]. Through inducing aberrant covalent bonds between adjacent pyrimidines, UVB generates mutagenic products, whilst UVA is less mutagenic and mediates indirect DNA damage by formation of hydroxyl and oxygen radicals through a photo-stress-mediated mechanism [323]. With a wavelength range that lies between 290 and 320 nm, solar UVB is only partly filtered out by the atmosphere and therefore can cause serious mutagenic changes to keratinocytes. UVB not only initiates dysregulation of tumour suppressor genes causing genomic instability but also promotes proliferation of modified cell clones which may result in malignant transformation of tissues [324].

UVB induces epidermal actinic keratosis which is a potentially malignant lesion of the skin; when the vermillion zone of the lips is affected, the lesion is termed actinic cheilitis [325]. Although clinical features of actinic cheilitis may not be present at the surgical margins of lip tumours, most lip cancers are preceded by this potentially malignant lesion [326]. This clinical evidence, in addition to multiple records of mutations reported in lip cancers [327, 328], further emphasises the role of solar UV exposure in the aetiology of lip cancer.

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Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy reported worldwide, with an estimated global burden of approximately 550,000 incident cases and 300,000 deaths per year and a high case fatality rate. Squamous cell carcinoma of the head and neck (HNSCC) is an anatomically heterogeneous group of neoplasms arising from the mucosal surface of the lip, oral cavity, oropharynx, hypopharynx, larynx, nasopharynx, tonsils, and larynx. Each year approximately 263,000 cases of oral cavity cancer and 135,000 cases of pharyngeal cancer are diagnosed worldwide [20]. Oral squamous cell carcinoma (OSCC) and oropharyngeal squamous cell carcinoma (OPSCC) are the most common types of HNSCC. Approximately 50 % of HNSCCs are in the oral cavity, followed by 30 % in the larynx and 10 % in the oropharynx. Alcohol consumption, smoking, smokeless tobacco, poor oral hygiene, and genetic features are key risk factors for HNSCC development [51]. In addition, in the last decade it has become clear that a subset of HNSCC covering approximately 25 % of the worldwide cases is associated with certain HPV types.

Human papilloma viruses [HPVs] are a large group of viruses belonging to the *Papillomaviridae* family, which are non-enveloped, have a circular genome of double-stranded circular DNA of 8 kb size coding for nine genes, are enclosed in an icosahedral capsid, and have a virion size of 50–55 nm diameter. A total of 204 subtypes of HPVs have been identified till date, which are categorized as high risk (HR) and low risk (LR) based on their oncogenic potential. A phylogenetic tree based on homologous nucleotide sequence of the major capsid protein- L1, classifies the different HPVs into five genera – alpha, beta, gamma, mu, and nu. They are strictly host specific and exquisitely tissue tropic, infecting only cutaneous or

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internal mucosal surfaces and hence being termed as epitheliotropic. Furthermore, HPVs are categorized as mucosal and cutaneous based on the type of tissue they infect.

In 1977, Harald zur Hausen hypothesized that HPV has a causative role in the cervical cancer [79]. In 1983 and 1984, zur Hausen and his collaborators identified HPV subtypes 16 and 18 in cervical cancer specimens [29], and in 1995, International Agency for Research on Cancer (IARC) accredited HPV16 as a carcinogen of cervix, uteri, and anogenital areas [35]. The association of HPV with oropharyngeal carcinoma was first proposed by 1983 by Syrjanen et al. [73] and was further confirmed through several studies by various research groups across the globe. A decade later after its recognition as a carcinogen of cervix and anogenitals, in 2007 IARC recognized HPV as carcinogen of oropharyngeal squamous cell carcinoma and possibly the oral cavity too [36]. Since then several studies have convincingly proved that HPV, known to be an etiological agent for genital cancers for long, is also a possible contributory agent for head and neck cancers. However, unlike the cervical cancer, high-risk HPVs in head and neck cancers are neither necessary nor a sufficient cause of cancer and only about 20 % of the HNC cases are associated with viral infection.

This chapter attempts to summarize the current knowledge of HPV's association with anatomical sites in the head and neck region, its role as a potential etiological agent for various head and neck cancers, various detection methods, clinical outcome, existing prevention strategies, and finally future research directions.

3.1 HPV Genome and Proteome

As indicated earlier, the genome of HPV is very small with just 7200–8000 base pairs and is organized into three distinct regions – early regions, late regions, and regulatory region. Early region genes encode for proteins E1, E2, E4, E5, E6, and E7, which are expressed during early stages of viral replication, while the late region encodes L1 and L2 proteins which are essentially the capsid proteins required only during viral encapsulation and shedding. The long control region (LCR) contains the regulatory elements for transcription and replication [55] and is also termed as URR – upstream regulatory region (Fig. 3.1).

Early viral protein E1 is necessary for viral DNA replication and helps in maintaining the episomal copy numbers with in the cell. E2 initiates the DNA replication process in association with E1 protein and also codes for proteins that regulate viral DNA transcription. E2 also plays an important role in cell transformation, inhibition of apoptosis, transcriptional regulation, and modulation of the immortalizing and transformation potential of HPV [53].

E4 protein is translated from a spliced E1/E4 mRNA transcript and is a cytoplasmic protein that disrupts the structural framework of keratin resulting in the thickening of the spinous and horned layer of the epidermis and the koilocytosis of the epidermis. Koilocytosis is a typical manifestation of HPV infection that describes the presence of koilocytes in the specimen. Koilocytes are squamous epithelial cells that

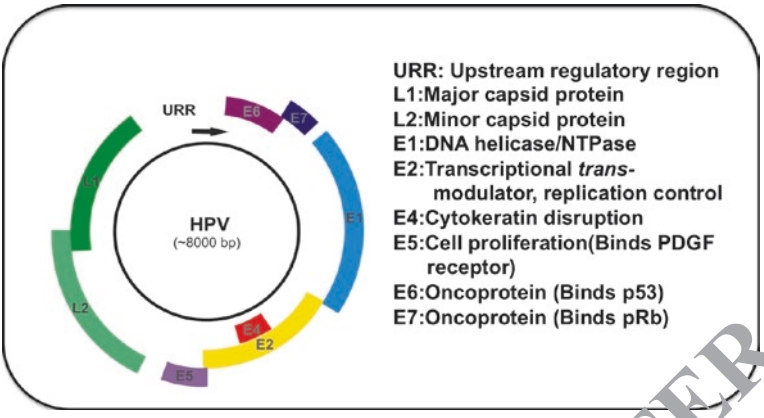

Fig. 3.1 HPV genome

undergo a number of structural changes such as nuclear enlargement, irregularity of nuclear membrane contour, hyperchromasia, and appearance of a perinuclear halo. This is typical to HPV-infected cells and forms an important criterion for Papanicolaou test (Pap smear test). E4 is overexpressed in cells supporting the viral genome amplification and is visible during advanced stages of the infection. As E4 is primarily overexpressed during the time of genome amplification initiation, it helps in staging of the tumor. In the later stages, E4 disrupts the cellular keratin network and accumulates cornified envelope that expedites release of the virus and its transmission [53].

E5 also is involved in the transformation process and helps in viral DNA replication. More importantly, E5 is a vital player in immune evasion strategies of HPV. E6 and E7 primarily impair cell cycle regulation and inhibit apoptosis and are hence the major oncoproteins of HPV playing a major role in HPV-dependent malignant transformation, the process that is explained in a later section [53].

L1 is the major capsid protein, while L2 is the minor capsid protein. The stoichiometry of L1:L2 in purified L1–L2 complexes is 5:1, indicating that a single molecule of L2 interacts with an L1 pentamer. The HPV capsid consists of 360 copies of the major capsid protein, L1, arranged as 72 pentamers on a icosahedral lattice, with substoichiometric amounts of the minor capsid protein, L2 [22]. The major capsid protein L1 produced through a recombinant DNA technology process inside a prokaryote can self-assemble into virus-like particles (VLP) and is the basis for assemblies of HPV vaccines, which can induce the production of neutralizing antibodies when administered.

3.2 Biology of HPV

Unlike most of the viruses, which generally infect and produce progenies from the same target cell, HPV life cycle requires the infected cell to undergo mitosis and differentiation. The life cycle of HPV is tightly linked to differentiation state of the

host cell and hence actively dividing host cells are a prerequisite for establishing HPV infection. Upon infection, the cells undergo incessant proliferation by overriding the cell cycle checkpoints and their machinery. This helps in viral DNA replication synchronous to the host DNA [18].

Biology of HPV-induced carcinogenesis is widely studied and described in the perspective of cervical cancer. There are four steps in the HPV-induced cervical cancer development – (a) HPV infection of metaplastic epithelium at the cervical transformation zone, (b) HPV viral persistence, (c) progression of the persistently HPV-infected epithelium to precancerous lesions, and (d) invasion through basement membrane of the epithelium. Since basal cells are the only proliferating cells in the normal epithelium, HPV infects them when they get exposed through the micro-abrasions in the epithelial surface. HPV genome does not code for any enzymes needed for viral replication; instead they utilize the host cell replication machinery. The viral genome is maintained at the basal layer of epithelium. As basal epithelial cells differentiate, the viral life cycle goes through successive stages of genome amplification, virus assembly, and virus release with a concomitant shift in expression patterns from early to late genes, including L1 and L2, which assemble into viral capsids. HPV infects epithelial tissue through exposed basal keratinocytes found in basal layer of the skin (stratum germinativum) following micro-abrasions of skin as would occur after a sexual intercourse (Fig. 3.2).

The epithelium of cervix is varied. The ecto-cervix (more distal, by the vagina) is composed of nonkeratinized stratified squamous epithelium. The endo-cervix (more proximal, with in the uterus) is composed of simple columnar epithelium.

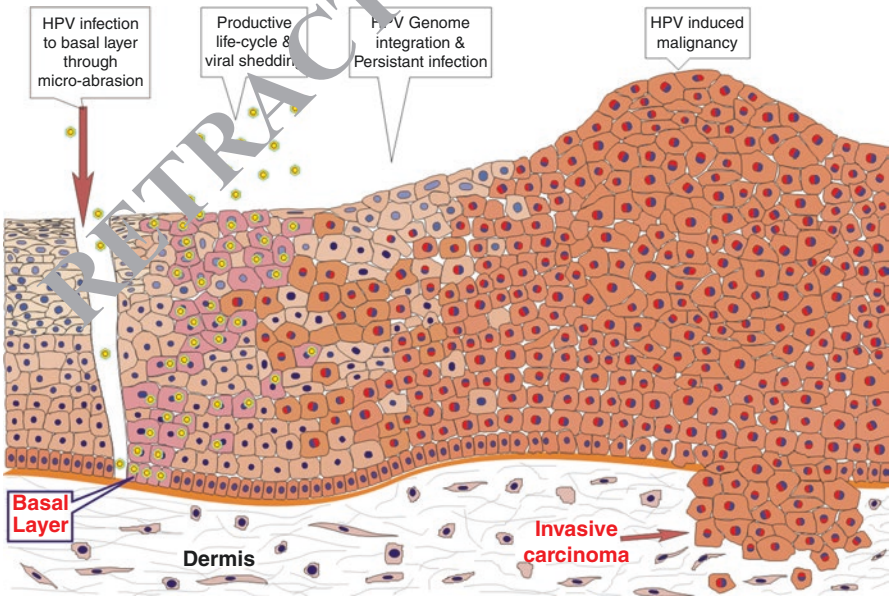

Fig. 3.2 HPV infection initiation and progression

The area adjacent to the border of endo- and ecto-cervix is known as the transformation zone. The transformation zone undergoes metaplasia numerous times during a lifetime. Metaplasia is the reversible replacement of one differentiated cell type with another mature differentiated cell type. When the endo-cervix is exposed to harsh acidic environment of the vagina, it undergoes metaplasia to squamous epithelium, which is better suited to vaginal environment. Similarly, when the ecto-cervix enters the less harsh uterine area, it undergoes metaplasia to become columnar epithelium. There are several instances when the metaplasia of the transformation zone occurs: puberty, when endo-cervix moves out of the uterus, changes associated with normal menstrual cycles, and postmenopause, when the uterus shrinks moving the transformation zone upward. These events of metaplasia in the cervix increase the risk of cancer in this area.

The human oral tract has a divergent histology of the mucous lining with columnar epithelium throughout the respiratory tract and stratified columnar epithelium covering the mucosa of the pharynx and larynx. The resulting squamocolumnar junctions (SCJs) can be compared to a similar junction in the cervix and could favor establishment of HPV infections. There is also evidence of HPV infection of gingival tissue [48]. Periodontal pocket – the only location of the gingival mucosa where basal cells are exposed to the environment – enlarges during progression of periodontitis as a result of chronic inflammatory processes [73]. The chronic inflammation results in increased basal cell proliferation leading to higher viral load in saliva as well as higher risk of HPV transmission [70]. Chronic inflammation and continuous epithelial proliferation in the junctional gingiva could thus favor the replication of HPV and might be an important reservoir for HPV in the oral mucosa.

3.3 Molecular Pathology of HPV Carcinogenesis

As in the case of anogenital HPV infection, oral HPV infections are also asymptomatic and usually cleared by itself by the normal immune system, within a year. Persistence of infection without clearance marks the first step toward HPV-induced carcinogenesis. The process of HPV-induced cell transformation is a combined manifestation of several discrete cellular, genetic, and molecular alterations accumulated in the mucosal tissue, termed "condemned mucosa syndrome," which later progresses onto invasive cancer [60]. HPV oncoproteins E6 and E7 are the key players in the process of oncogenesis. They, along with the supporting role from other early proteins, promote the uncontrolled proliferation of HPV-infected cells (Fig. 3.3).

Upon HPV integration, E2 gene promoter gets deleted and results in the transcription of E6 and E7 genes. E7 binds to retinoblastoma (Rb), a tumor suppressor protein, and other members of the Rb family, such as p107 and p130. Proteins from the Rb family regulate the G1–S-phase transition through interaction with the E2F family of transcription factors, which in turn control many genes that are involved in regulating cell cycle progression, differentiation, mitosis, and apoptosis. Binding of the E7 oncoprotein to the Rb protein leads to Rb protein degradation by

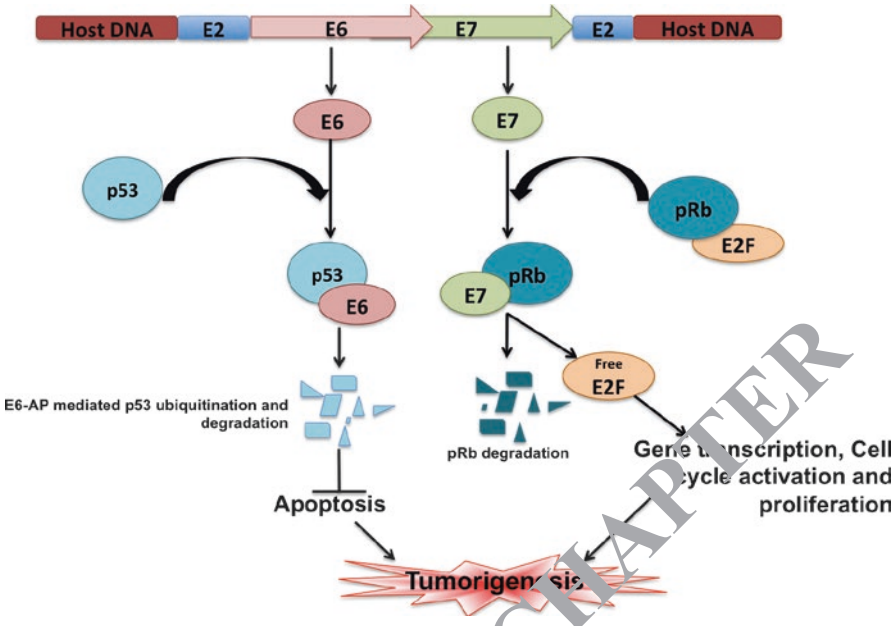

Fig. 3.3 Molecular pathology of HPV E6/E7-mediated tumorigenesis

ubiquitination and E2F is released. The E2F thus released activates genes such as cyclin A and cyclin E and promotes the entry of cells into S phase. This would also lead to the compensatory overexpression of both cytoplasmic and nuclear p16 protein in HPV-infected tumor cells, which is used as a biomarker for HPV-associated lesions and cancers. The p16 tumor suppressor gene is a member of the INK4 class of cell cycle inhibitors and represents a key component of the Rb pathway. p16 protein binds to cyclin-dependent kinases 4 and 6, thereby blocking its interaction with the D-type cyclins. This maintains the retinoblastoma (Rb) gene in a hypophosphorylated state that binds E2F transcription factor trying to prevent cell cycle progression [18].

Abnormal cell proliferation and DNA synthesis in the absence of sufficient growth signals due to HPV infection in cells can activate p53-dependent apoptotic programs. The inactivation of Rb by E7 protein sensitizes cells to p53-dependent apoptotic signals, but high-risk HPV protein E6 protein targets p53 for degradation, thus inhibiting the proapoptotic functions of p53 [67]. Under normal circumstances, p53 arrest the cells in G1 phase and induce apoptosis or repair of DNA damage. However, HPV E6 deregulates this mechanism and genomic instability of the infected cell results. The virally encoded E6 binds to a cellular ubiquitin/protein ligase, E6-associated protein (E6-AP), and p53 resulting in ubiquitination of p53 leading to its proteolytic degradation. Furthermore, E6 and E7 also interfere with growth inhibitory cytokines such as tumor-necrosis factor-α (TNF-α). TNF-α activates the extrinsic apoptotic pathway through TNF receptor 1 (TNFR1), Fas cell surface death receptor (FAS), and the TNF-related apoptosis-inducing ligand

(TRAIL) receptors. E6 abrogates the apoptotic effect of TNF-α by binding to TNFR1, which inhibits the subsequent transduction of apoptotic signals [21].

Apart from its action on p53, E6 also disrupts the mitochondrial apoptotic pathway by interacting with proapoptotic Bcl2 family members BAK and BAX as well as by inducing expression of inhibitors of apoptosis proteins (IAPs) and Survivins [26]. The expression of E6 and E7 can result in immortalization of host cells, induce genomic instability in them, and result in mitotic defects such as multipolar mitoses, anaphase bridges, and aneuploidy. E6 and E7 also induce DNA damage and increase the frequency of foreign DNA integration into the host genome. Although under normal circumstances, cells with mitotic defects are targeted for cell death, and the E6 and E7 act on cell cycle checkpoints and apoptosis, which allow these abnormal cells to survive and accumulate.

As most people with HPV infection do not develop cancers, expression of E6 and E7 is necessary but not sufficient for malignant transformation. However, increased proliferative capacity and evasion of apoptosis induced by E6 and E7 can lead to the accumulation of DNA damage and mutations that can ultimately result in malignant transformation and carcinogenesis. E7 protein of high-risk HPVs has the capacity to reprogram terminally differentiated epithelial cells of the surface epithelium and encourage host cells to reenter the cell cycle and sets stage for viral DNA replication.

3.4 HPV-Associated Oral Diseases: Diverse Manifestations

HPV infections in the oral region range from asymptomatic to visible lesions, which can be benign or malignant. Disease manifestations due to HPV infection of the oral region include:

(a) *Condyloma acuminatum*

Oral condylomata are typically papillary proliferations of squamous epithelium with prominent acanthosis and parakeratin that line deep crypts, similar to their counterparts in the lower genital tract. Koilocytic features are most prominent toward the surface of the lesion. The lesion is caused by the abnormal proliferation of a squamous stratified epithelium. Sexual contact remains the usual route of transmission (20 %) and people who practice oral sex have a 50 % chance of acquiring oral condyloma. Condyloma acuminatum has tropism to the tongue, lips, palate, and mouth floor and is seen as little pinkish or whitish nodules which proliferate in papillary projections that might be either pedicle or sessile. Outline surfaces present even more evident cauliflower shapes than papilloma, mainly when they converge. HPV6 and HPV11 are mostly involved and 75–85 % positivity has been observed [10, 11, 46].

(b) *Verruca vulgaris (common wart)*

Verruca vulgaris is also known as common wart and one of the most common lesions affecting children usually on the lips, hard palate, and gingival and tongue dorsal surface. Verruca vulgaris show an almost symmetrical structure,

with elongated rete ridges that are shorter at the periphery than in central area. Thin elongated connective tissue papillae form papillomatosis. The cryptoform surface shows a conspicuous hyperkeratinization. HPV presence of up to 100 % has been observed in these types of lesions. Most oral warts are self-limited and resolve within a couple of years [10].

(c) *Oral squamous papilloma*

Oral squamous papilloma (OSP) is a benign lesion. Though observed in patients of all ages, it more commonly affects adults between 30 and 50 years. HPV6 and HPV11 are widely associated with these tumors. While the lesion is usually located in the oral mucosa, mostly on the palate and tongue in adults, in children the laryngotracheobronchial complex is a more common site. OSP affects the soft palate, the lingual frenulum, as well as the lower lip and the uvula, most often presenting as a single, small lesion of size <1 cm, with exophytic growth and a wide basis or pedicle. Epithelial proliferation patterns are similar to previous lesions, with squamous cell acanthosis, hyperkeratosis, and a centrally disposed fibrovascular core. Koilocytosis may or may not be present [10, 11].

(d) *Focal epithelial hyperplasia (Heck's disease)*

Focal epithelial hyperplasia (FEH) or Heck's diseases is a benign epithelial growth that commonly affects the oral mucosa, lips, and tongue, particularly the lower lip and more rarely the palate, floor of the mouth, and oropharynx. Multiple papules of 3–10 mm that get converged are typical to FEH. They are characteristically nodular, sessile, circumscribed, painless, and soft masses on oral mucosa and seen in pale pink color. It affects all age groups but it is more common in children and adolescents. FEH exhibits epithelial hyperplasia, acanthosis, mild parakeratosis, and anastomosing rete ridges. Superficial layers of the epithelial tissue contain koilocytes and apoptotic or dyskeratotic cells. FEH has a steady association with HPV infection, and the most common types are 13 and 32, contributing to 90 % of infections. Though HPV1 and HPV11 rarely show potential for malignancy, malignant transformation has been reported only with HPV24. FEH normally regresses spontaneously in a few months or years [7, 10, 17].

(e) *Oral lichen planus*

Oral lichen planus (OLP) is disease affecting the skin and the mucosa and seemingly interconnected to HPV infection. OLP mainly affects female populations between 30 and 60 years. Both oral and genital mucosae are affected. OLP lesions are usually bilateral and symmetrical affecting the oral mucosa, gingiva, as well as dorsum of the tongue and the lip mucosa. The lesions may be single or multiple and may present in a wide range of forms – cauliflower-like, striated, or annular. OLP lesions are painful and presented as reticular, erosive, and atrophic forms. They have typical acanthosis in keratotic lesions, atrophy in older lesions, hydropic degeneration of the basal layer, as well as a strong

subepithelial lymphocytic infiltrate. Those lesions that present dysplasia are not classified as OLP. The most prevalent and commonly found HPV subtypes in OLPs are HPV11 and HPV16 [11].

(f) *Oral leukoplakia*

Leukoplakia is considered a premalignant lesion or potentially malignant disorder in oral cavity, which can later transform into oral squamous cell carcinoma (OSCC). Smoking and chewing tobacco along with alcohol consumption are the main risk factors, while candidiasis, HPV, and Epstein-Barr virus act as cofactors. HPV6, HPV11, and HPV16 have been predominantly found, while HPV18, HPV31, HPV33, and HPV35 are found more rarely in the lesions. HPV16 is the most prevalent subtype in oral leukoplakia. Oral leukoplakia (OL) is mostly located on the lip vermillion, gingiva, tongue, and floor of the mouth with epithelial changes ranging from innocuous hyperplasia to dysplasia of varying degrees. OL presents hyperkeratosis and epithelial hyperplasia without dysplasia. Depending on the dysplasia degree, it may be classified as low, intermediate, and high risk of malignancy to predict the malignant transformation [11, 50].

(g) *Oral verrucous carcinoma*

Oral verrucous carcinoma (OVC) is another squamous cell carcinoma (SCC) subtype often with less aggressive behavior and can be located on the head, neck, and genitals and more notably on the oral mucosa. OVCs are rather rare and HPV subtypes 6, 11, 16, and 18 have been widely associated with it. They are predominantly seen in 50–80 year-old males in the oral mucosa, gingiva, mandible alveolus crest, tongue, and lips. They appear with slow exophytic growth, resulting in verrucous cauliflower lesions; with white plaques, normally extensive; and with well-demarcated hyperkeratotic lesions. OVCs also present with acanthosis and keratinization with keratin plugging and clefting. They are irregular with minimal atypia and usually there is inflammatory infiltrate on the subepithelial layer around epithelial invaginations that compress the underlying tissue. Since they share clinical feature of oral squamous cell carcinoma (OSCC), often it is difficult to distinguish OSCC from OVC. Moreover, tumors mainly composed of OVC may contain small areas of OSCC and behave as one [66].

(h) *Oral/ oropharyngeal squamous cell carcinoma (OSCC/OPSCC)*

OSCC represents 90 % of all oral cancers and most commonly affect the tongue, especially on inferior and lateral surfaces, and also the buccal mucosa, lips, posterior mandibular ridge, gingiva, hard palate, and retromolar trigone, while oropharyngeal carcinoma affects the oropharynx, tonsils, etc. They are usually nodular or ulcerative lesions with exophytic or ulceroproliferative features. In these lesions, the epithelium invades into the stroma that may occur as islands, cords, sheets, and isolated epithelial malignant cells. Keratin may be present, mostly in well- and moderately differentiated tumors. There are varying degrees of atypia and nuclear and cellular pleomorphism with aberrant and

regular mitosis. HPV16 predominates among the HPV-positive head and neck cancers with 90 % positivity and 50 % in oropharynx. Potentially malignant manifestations like leukoplakia, erythroplakia, proliferative verrucous leukoplakia, and lichen planus may gradually progress to OSCC and oropharyngeal squamous cell carcinoma.

3.5 Epidemiology of HPV in HNSCC

The epidemiology of HNSCC has been comprehended to be transforming dramatically over past 25–30 years. Despite decline of the use of tobacco in several countries due to improved awareness as a potential carcinogen, oropharyngeal cancer incidence rates demonstrate an increasing trend. Upon deeper investigations, it was observed that the HPV-unrelated oropharyngeal cancer has decreased corresponding to the decrease in the tobacco use, while the increased incidence of HPV-associated oropharyngeal cancer was responsible for overall increase of HNSCC rates. This realization advocates the importance of HPV as an etiological agent of oropharyngeal as well as other HPV-infected subsites of the head and neck area. This increased prevalence rates could possibly be due to one or combination of four different reasons: (a) the knowledge of HPV association with oral cancer has led to several research groups working on the issue and more results from various areas coming up, (b) improved HPV detection methods with increased sensitivity and specificity, (c) false-positive results through cross contaminations due to increased specificity of the detection methods, and (d) an actual increase in the prevalence of HPV-associated oral cancers.

The HNC subsites can be arbitrarily divided into three groups: HPV associated, potentially HPV associated and potentially HPV unrelated. The tonsils, Waldeyer's ring, base of the tongue, lingual tonsil, and oropharynx belong to the first group. The tongue, larynx, and oral cavity belong to the potentially HPV-associated group. All the remaining HNC sites are grouped into the potentially HPV-unrelated group. This classification is based on the data from several previous studies associating HPV and site of tumor.

In HNC, the majority of the HPV-associated cases (between 86 and 95 %) are associated with HPV16 – the major high-risk oncogenic HPV [44]. Surprisingly, HPV6, which is considered to be a low-risk HPV, is seen in greater number of HNCs than any of the oncogenic types. Another low-risk subtype, HPV11 has also been detected to a large extent of HNCs suggesting that both HPV6 and HPV11 may not be benign in this anatomical locale [59].

Risk factors such as younger age patients with multiple lifetime sex partners, practice of oral-genital sex and/or oral-anal sex, use of oral contraceptives, and a history of genital warts could be some of the factors favoring transmission of HPV to the oral cavity and subsequent development of HPV-associated oropharyngeal cancers. It has been reported that not only oral sex but also open-mouthed kissing could result in acquiring oral HPV infection [15]. The excessive incidences of tonsillar and tongue cancer in the husbands of cervical cancer patients also confirm the

role of sexual behavior in oral HPV infection [31]. The increased incidence of HPV in the younger age group could be attributed to changes in sexual behavior patterns of newer generations, i.e., multiple oral sex partners or debut of oral sex at an early age. It is also noticed that oral HPV infections are more frequent in men than in women. It is speculated that this could be attributed to higher prevalence of HPV in the female genitalia rather than penile tissue, enhancing the chances of HPV infection while performing oral sex on a woman, thus contributing to the increase of acquiring oral HPV infection in men. Interestingly, no oral HPV infection has been detected among homosexual women, virgins, or women where sexual exposure was unknown suggesting that oral HPV infection may be more likely associated with sexual exposure to male partners rather than females [62]. It is a fascinating observation that homosexual women have no HPV if they practice oral sex, as is common among the group. It is rather intriguing that if men get HPV through oral/genital/anal sex, what prevents women from getting the infection, if they also have similar practices. Moreover, no significant difference has been reported in the prevalence of HPV infection between heterosexual and bisexual women. However, an increased prevalence of oral HPV infection is observed in women who had vaginal sex exposure but no oral sex exposure, which implies that vaginal sex exposure may be a stronger predictor for oral HPV infection [63]. Currently, not much is known about oral HPV infection and oral cancer in homosexual men (males having sex with males – MSM). There are reports that MSM are more likely to develop HPV-related anal cancer than men who only have sex with women, but it has not been established what the risk for oral cancer is in MSM. A prevalence positivity of 2–17 % has been reported in MSMs in various studies, which gets considerably increased if the immune system of the subjects is compromised by being HIV positive [43, 54, 63, 75]. HPV type 16 was the most frequently detected high-risk type with a prevalence of 2.0 %, while HPV type 6 was the most prevalent low-risk type. However, MSMs should be concerned about oral cancer for three reasons: (1) oral cancer is increasingly being found in younger men, (2) this increase is thought to be related to oral sexual behavior, and (3) the number of MSM with HPV infection or anal cancer is high.

Although studies suggested that sexual behavior plays an important role in HPV-positive HNC, a considerable number of HPV-positive cases appear to be independent of this risk factor [51]. The risk factors and natural history of oral HPV infections are largely unknown, in contrast to very detailed understanding of HPV infection in the female genital tract.

Of the several factors, synergistic effect between HPV and alcohol in the oral carcinogenesis is noticeable, while association observed between tobacco and HPV was insignificant. Among heavy alcohol users detected with the virus, the risk of head and neck cancer was statistically significantly increased relative to that of HPV-negative cancer drinkers. Alcohol can biologically modify mucosal tissue, potentially increasing its permeability to viral infection, or it could influence the immune response to HPV [69].

The overall incidence of HNC is decreasing in western countries due to the awareness of the adverse effects of tobacco in human carcinogenesis. However, the

percentage of oropharyngeal cancers is steadily increasing in the USA and Europe [4, 12, 51, 57, 71]. Epidemiological studies have shown that the fraction of oropharyngeal cancer linked to viral infection can substantially vary in different countries and over time. In the USA, the percentage of HPV-associated oropharyngeal cancers reported through different studies ranges from 40 to 80 % [51, 65]. The increase in both population-level incidence and survival rates of oropharyngeal cancers in the last three decades is attributable to HPV association with cancer cases.

Similarly, in Europe it has been reported that the proportion of HPV-positive cases can vary between 90 % in Sweden and less than 10 % in countries with heavy tobacco consumption [51]. This variation in the percentage of the HPV-positive cases could be explained by variation of incidence and prevalence of oral HPV infections between countries and/or by variation of risk factors other than viral infection. However, it is also possible that in some cases differences between studies are due to differences in sensitivity and specificity of the analytical techniques used.

In Sweden, HPV-positive tonsillar cancers almost doubled for each decade between 1970 and 2007. The proportion of HPV-positive tonsillar squamous cell carcinoma in Sweden was approximately 20 % in 1970, while it increased to 57 % in 1990 and reached 93 % in 2007 [57]. Incidences of HPV-related and potentially HPV-related sites have increased during past three decades in Denmark, especially in men, whereas those cancers at sites probably not HPV related decreased in men or showed virtually no changes in women. The largest increase in incidence was observed in the tonsillar cancer with a fourfold increase from 1978 to 2007 [8]. Likewise, an increase in the fraction of HPV-associated oropharyngeal cancer has been observed in France also [40]. In Finland the age-standardized incidence rate of cancer of the palatine tonsils doubled between 1956 and 2000 [71]. In Australia though the prevalence of oral HPV infection is around 2.3 % [3], in head and neck cancer cases, there is 36 % HPV positivity. HPV-related cancer was found to increase from 19 % (1987–1990) to 47 % (2001–2005) [34].

The Indian subcontinent has the highest HNC incidence in the world and accounts for one-third of the world burden [20]. HNC accounts for approximately 30–40 % of all cancer types in India. Many etiological factors are involved in HNC development in the Indian population, including alcohol and smoking. The habit of chewing betel quid often mixed with tobacco is widespread in Southeast Asia and also plays a key role in HNC development [24]. However, only a few studies have analyzed the presence of HPV infection in HNC in the Indian population [6, 14, 27, 56]. Prevalence percentage of HPV DNA in these studies varied from 27.5 % [27], to 74 % [6]. However, incorrect sampling, outdated methodology, and frequent cross contaminations in the laboratory may also be a serious compounding factor in these varied results.

Whereas the majority of HPV-driven cancers of the head and neck are oropharyngeal squamous cell carcinoma (OPSCC) comprising the tonsils and base of the tongue [16, 57], it is still in debate whether HPV may also have a causative role in other HNSCC subsites. The presence of HPV is found to be highest in the tonsils with 79 % and in base of the tongue with 73 %. There are solid indications that incidence and prevalence of HPV-associated HNSCC are increasing which are

particularly discussed to be correlated with a decline in smoking habits [38, 42]. Recent publications showed an increased incidence of HPV infections in HNSCC of approximately 50 % [12, 49] with HPV16 being the most prevalent type in at least 90 % of this cancer [44].

3.6 HPV-Associated HNC: Clinical Outcome in an Altered Entity

HPV-positive head and neck cancers are morphologically, genetically, and pathologically a distinct entity when compared to their negative counterparts (Table 3.1). Morphologically, HPV-positive HNCs are basaloid and show lobular growth, with no dysplasia of surface epithelium but without significant keratinization, whereas HPV-negative HNCs are moderately differentiated and keratinizing. HPV-positive and HPV-negative HNCs also manifest distinct transcriptomic patterns. This could be due to the HPV E6- and E7-mediated alteration in the genome and epigenome.

Evidence indicates that the classification of HNC as HPV-positive or HPV-negative lesions is very crucial for HNC treatment and prognosis. Indeed, patients with HPV-positive oropharyngeal cancers appear to have higher response rates to radiation and chemotherapy and increased survival than the ones with HPV-negative tumors [51] (Fig. 3.4). This phenomenon could be due to the fact that, in HPV-positive cancer cells, a reversible process inactivates the functions of several cellular tumor suppressors mediated by E6 and E7 oncoproteins. For instance, in HPV-infected cells, in contrast to a large proportion of HPV-negative HNC, p53 gene is not mutated, but E6 oncoprotein promotes the degradation of its product via the proteasome pathway leaving the p53 gene intact [28]. It is likely that in the presence of cellular stress, such as high doses of radiation, p53 functions are regained in HPV-positive cells leading to apoptosis. In the majority of the HNC studies, HPV positivity has been exclusively evaluated by HPV DNA detection assays. A few studies have also analyzed the expression of HPV oncogenes E6 and/or E7 in HNC lesions and showed that a significant proportion of HPV DNA-positive cases were negative for E6 and E7 expression [9, 40]. Whether prognosis of HPV DNA-positive and RNA-negative HNC is similar to HPV DNA- and RNA-positive cases remains

Table 3.1 General characteristics of HPV-positive HNCs

1.	Lower mean age of the patients
2.	Lower or no involvement of classical risk factors such as smoking and alcohol consumption
3.	Distinct sexual behaviors: higher number of partners and oral sex practice
4.	Aggressive metastasis to the lymphatic system of the neck
5.	Better response to radio- and chemotherapy as compared with HPV-negative tumors
6.	Better prognosis (less severe course and longer survival time)
7.	Low number of DNA mutations
8.	Single small chromosomal aberrations
9.	Long promotion (latency period)

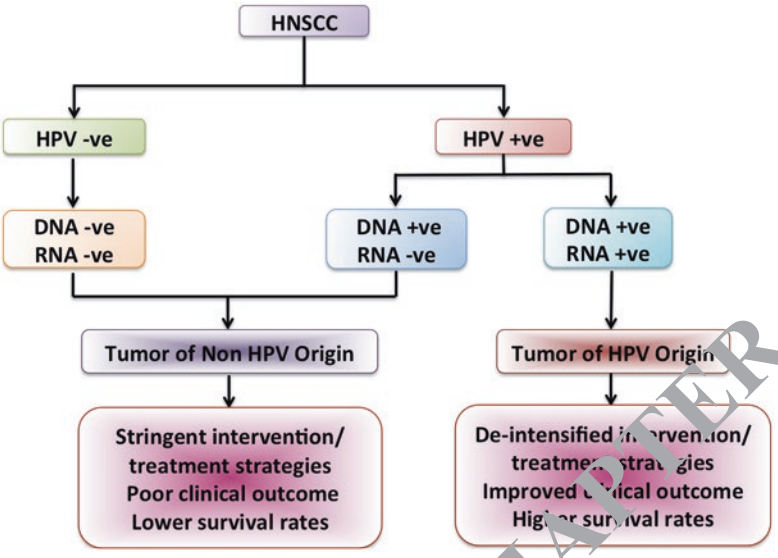

Fig. 3.4 HNSCC classification according to HPV tumor status

an open question. The epigenomics of HPV-positive and HPV-negative HNCs are considerably different. HPV-positive tumors are driven by methylation patterns in the promoter region than HPV-negative tumors (Table 3.2). Furthermore, HPV-positive tumors showed increased methylation in genes involved in activating invasion and metastasis [74]. A detailed characterization of HPV-positive cases by different parameters combined with follow-up studies will provide additional insights on therapeutic strategies for HNC.

There could be several explanations for the better outcome observed in patients with HPV-positive HNC. Factors intrinsic to individual tumors (e.g., specific mutations, HPV status, etc.) can play a major role in modulating the tumor microenvironment, which can influence immune cell infiltration, stromal architecture, and tumor vasculature, among several other factors. Being a cancer of viral origin, body's immune system plays a very important role in combating HPV-positive cancers due to the expression of viral proteins within HPV-positive HNC.

Presence of HPV-specific T cells and a greater shift from naïve to effector and memory T cells in the HPV-positive vs HPV-negative HNC patients all advocate a better immune response to HPV-positive tumors. An acceptable explanation for the dissimilarities in treatment outcome between HPV-positive and HPV-negative patients might be that virally driven tumors evoke an adaptive immune response against viral antigens expressed by the tumor. HPV16-specific $CD8^+$ T cells have been detected in the blood of HPV-positive oropharyngeal squamous cell carcinoma (OPSCC) patients and isolated from tumors as well, implicating a role for the anti-tumor immune response [2, 33, 76]. Seropositivity to E6/E7 provides better survival in HPV-positive patients [47]. Moreover, infiltration of HPV-positive HNSCC by

Table 3.2 Key genes altered in the HPV-positive tumors

Gene/protein	Function
Genes with promoter methylation	
CCNA1	Cell cycle regulator
GRB7	Cell growth and migration regulator
SYBL1	MT-1 MMP-dependent matrix degradation
TIMP3	Inhibition of tumor growth and angiogenesis
SFRP4	Wnt pathway antagonist
CDH11	Cell-cell adhesion
JAK3	Cytokine receptor–mediated intracellular signal transduction
TUSC3	Glycosylation efficiency regulator
RUNX1T1	Interaction with DNA-bound transcription factor
TCF21	Cell fate differentiation
IRX4	Interaction with Vitamin D receptor
GATA4	Cell differentiation promoter
GFRA1	RET tyrosine kinase receptor activator
Genes upregulated	
CDKN2A	Cell cycle inhibitor, product of E2F transcription
PCNA	Cell cycle–dependent auxillary protein for DNA polymerase
RFC4	Accessory protein with PCNA for DNA pol delta and epsilon
MCM2	Early S-phase protein involved in DNA replication
MCM3	Helicase that links a histone chaperone to a histone H3–H4 bridge and involved in DNA replication
CDC7 (cell division cycle-7)	Protein kinase for G1/S transition and initiation of DNA replication
TYMS (thymidylate synthetase)	Maintains the dTTP pool for DNA replication and repair
CCNE2	Activates CDK2 and is involved in DNA synthesis
USP1	Negatively regulates PCNA polyubiquitination
BRG1	Involved in ATP-dependent chromatin remodeling mechanism in the mitotic division

programmed death-1 (PD-1)-expressing T-lymphocytes has been shown to be a favorable prognostic factor [5]. High levels of tumor-infiltrating lymphocytes (TILs) have also been identified as a favorable prognostic biomarker in HPV-positive tumors. TILs permeate HPV-positive OPSCC and confer a protective effect through an adaptive host immune response directed against viral antigens [77].

Survivin belongs to inhibitor of apoptosis (IAP) family with a bifunctional role that acts as a suppressor of apoptosis and plays a central role in cell division. Survivin mediates the regulation of both cell viability and cell division. The nuclear pool of survivin is likely to be involved in promoting cell proliferation, whereas the cytoplasmic pool of survivin may participate in controlling cell survival. Nuclear survivin is associated with HPV negative compared to HPV-related OPSCC and correlated with a poor clinical outcome in HPV-independent ones, suggesting a fundamental role in cell death regulation [61].

SMG-1 (suppressor with morphogenetic effect on genitalia) is a signaling protein that belongs to a family of phosphoinositide 3-kinase (PI3-kinase)-related

kinases (PIKKs) and is involved in the maintenance of genome integrity via genotoxic stress response pathways and plays an important role in the DNA damage response network. SMG-1 gene promoter gets hypermethylated and its expression is reduced in HPV-positive tumors. Low SMG expression correlated with HPV positivity status and improved patient survival, while decreased SMG-1 in HNSCC cells resulted in their increased sensitivity to radiation [30] suggesting that increased sensitivity of HPV-positive tumors to radiation may be due to the impaired ability of the cancer cells to respond to DNA damage. HPV-related HNSCCs have less frequent p53 or pRB mutations, which often occur in HPV-negative tumors [51]. The tumor suppressor p53 mediates the cellular stress response including DNA damage-induced apoptosis and cellular senescence by chemotherapy and ionizing radiation. Hence, the presence of wild-type p53 in HPV-related tumors could confer enhanced chemo- and radiosensitivity compared to HPV-negative tumors with p53 mutations.

HPV-positive HNCs are now accepted to be more radio- and chemo-sensitive, and hence strategies to de-escalate the treatment modalities that alleviate the treatment-associated morbidity are highly recommended for patients with HPV-positive HNCs. Therapeutic de-intensification by lowering the doses of chemo- and/or radiotherapy may be justifiable for the patients with HPV-positive HNCs that are less aggressive than their HPV-negative equivalents.

3.7 HPV Detection in Clinical Samples

The ultimate goal of any developing technology for HPV detection in clinical samples is to increase the sensitivity and specificity of detection while maximizing efficiency, simplicity, reproducibility, and transferability to a routine diagnostic laboratory. Notwithstanding the fact that HPV positive HNSCCs are a distinct entity with a higher prognostic advantage and a need for a consistent detection system for HPV from clinical samples, there is no standard strategy for HPV detection in head and neck cancer clinical samples. HPV testing methods vary considerably across laboratories reflecting the predispositions and tendencies of individual investigators. HPV detection strategies vary not only in design but also in detection targets, which include HPV DNA, post-integration transcription of HPV E6 and E7 mRNA, viral oncoproteins E6 and E7, and overexpression of cellular proteins such as p16- and HPV-specific serum antibodies. Many assays are available, including commercial kits, PCR-based methods, HPV-type-specific DNA in situ hybridization, serological assays, and immunohistochemical protocols to detect surrogate biomarkers of viral infection. For widespread implementation in the clinical arena, detection methods must be accurate, cost-effective, and readily transferrable to the routine diagnostic laboratory. A better characterization of HPV in HNC may also contribute in estimating the impact of prophylactic HPV vaccination on the future incidence on cancers other than those of the uterine cervix caused by the HPV [41].

An ideal detection test should have the ability to both recognize the mere presence of HPV in the tissue and recognize its potential to be a driving force of

tumorigenesis. A detection method would have no clinical significance if it cannot differentiate an incidental virus (e.g., viral contaminant) from an active oncogenic agent. A test that can detect the presence of the viral oncoproteins E6 and E7 is generally regarded as the gold standard method of clinically relevant HPV. Presence of E6/E7 mRNA is considered a surrogate to the presence of these proteins and is the current standard detection system. The ongoing challenge of HPV detection efforts has been to reproduce the accuracy and reliability of the PCR E6/E7 mRNA assay using techniques that are easier and transferrable to the diagnostic laboratory.

(a) *HPV DNA*

PCR amplification of HPV DNA allows the specific amplification of trace DNA sequences in a biological sample. The primer sets can either be designed to target highly conserved consensus sequences shared by multiple HPV types, thus allowing simultaneous identification of a wide range of HPV types, or they can be designed to target type-specific viral DNA sequences permitting HPV genotyping. PCR-based methods can detect HPV well below one viral copy genome per cell accounting for its incomparable sensitivity. The challenging factors in using PCR-based methods are that, first, clinical samples are very prone to cross contamination and, second, PCR-based methods do not permit the distinction between HPV that acts as a driver of malignant transformation and a transcriptionally silent virus that plays no role in the process of tumorigenesis (i.e., passenger virus). This shortcoming may be overcome through a real-time PCR approach that can better measure viral load [78].

(b) *HPV RNA*

Detection of E6/E7 messenger RNA (mRNA) is the current gold standard assay for clinically relevant HPV that recognizes the presence of HPV and discerns its potential as a driving force of tumorigenesis. The starting material can either be fresh or formalin-fixed paraffin-embedded (FFPE) tissue and, although has wider clinical applications, remains more restricted to research laboratories. It continues to be a challenge to reproduce the accuracy and reliability of the PCR E6/E7 mRNA assay using techniques that are easier to be carried over in a routine diagnostic pathology laboratory [78].

(c) *In situ hybridization*

In situ hybridization (ISH) utilizes labeled DNA or E6/E7 mRNA probes complementary to targeted viral sequences, binds to them, and amplifies the signals and permits direct visualization of viral transcripts in routinely processed tissues. The DNA probes may hybridize either to HPV-type-specific DNA sequences or to a consensus sequence shared by multiple HPV types or may be mixed in a single reaction to cover an extended range of HPV types (i.e., probe cocktail). It can be used for detection of HPV in both fresh and FFPE tissues. The various signal amplification steps have improved its sensitivity to extend of detecting as few as one viral copy per host genome. The development of nonfluorescent chromogens

allows visualization of hybridization using conventional light microscopy; thereby reducing the requirement of more sophisticated machinery makes this technique compatible with standard tissue processing procedures and thus widely transferrable to most surgical pathology laboratories. Testing for HPV E6/E7 transcripts by RNA ISH is an ideal platform for HPV detection in clinical samples and is the most direct evidence of HPV-related tumorigenesis by confirming the presence of integrated and transcriptionally active virus by permitting the visualization of viral transcripts directly in tissue sections [78].

(d) *p16 staining*

Immunohistochemical staining for HPV surrogate markers, such as $p16^{INK4a}$, is an attractive possibility. Considering HR-HPV E6/E7 mRNA expression as the gold standard for HPV status, both p16 immunohistochemistry (sensitivity, 96.8 %; specificity, 83.8 %) and HPV16 in situ hybridization (sensitivity, 88.0 %; specificity, 94.7 %) showed excellent performance in HPV detection [39]. Although $p16^{INK4a}$ detection has a high sensitivity, validation studies have shown that $p16^{INK4a}$ overexpression can be also detected in approximately 20–30 % of HPV-negative HNC [19, 68]. Ideally, a simultaneous analysis of two or more surrogate markers could improve the specificity of the assay. Several studies on *in vitro* experimental models have shown that HPV16 E6 and E7 have the ability to induce the upregulation of several cellular proteins, e.g., enzymes involved in the sumoylation machinery [52] and positive regulators of signaling pathways [37]. However, their use as surrogate HPV markers needs to be validated in clinical studies.

(e) *HPV serology*

All the detection systems mentioned above require high level of technical expertise and sophisticated laboratory set up. On the other hand, serological assays to detect antibodies against viral protein antigens can be relatively simple and easy to establish in clinical laboratories. It is now known that antibodies against HPV L1 represent cumulative past HPV infection from multiple possible anatomical sites (i.e., genital, anal, or oral) and do not imply the presence of an HPV-related tumor. Conversely, antibody markers against HPV E6 and E7 oncoproteins should occur in response to an underlying HPV-driven neoplastic process and would be expected at low levels among cancer-free individuals. Recent studies prove that HPV16 E6 seropositivity was present more than 10 years before diagnosis of oropharyngeal cancers, making it a good diagnostic marker for predicting HPV-induced oropharyngeal cancers [45].

3.8 HPV Vaccinology

HPV-associated cancer is a perfect candidate to be prevented through vaccination as evidenced by several efficacy studies of HPV vaccines in preventing cervical cancer. In the last decade, a vast number of studies validating prophylactic vaccines for

HPV16 and HPV18 have shown almost 100 % efficacy against the development of HPV16- or HPV18-associated cervical premalignant lesions [23, 25, 58]. The only study that has assessed the efficacy of HPV vaccine against the oropharyngeal HPV infections provided a proof of principle that HPV vaccine may prevent the HPV-induced oral and oropharyngeal cancers [32]. Along with the potential benefits of HPV vaccines directly against oropharyngeal HPV infection, decrease in genital HPV infection by vaccines may also indirectly reduce the oropharyngeal HPV infection, as they are largely associated with sexual behavior. It is likely that HPV vaccination may also prevent infection in the oral cavity [41], although to date there is no data to support this hypothesis. Due to the role of HPV infection in HNC, it is of paramount importance to evaluate the possible impact of HPV vaccination in the prevention of oral infections. Since the vast majority (>86 %) of HPV-associated HNC appears to be due to HPV16, the relative impact of efficient vaccination with the current vaccines targeting HPV16 on prevention of HPV-associated HNC could be even greater than in cervical cancer. These aspects are crucial for the development of novel HPV vaccination strategies that include also men, who appear to have a higher incidence of HNC in comparison to women and are not currently included in any national vaccination plans worldwide.

As of 2014, two HPV vaccines have market approval in many countries – Gardasil™ and Cervarix™. The FDA-approved and licensed quadrivalent vaccine Gardasil, produced by Merck-Sharp & Dohme Inc, contains recombinant virus-like particles (VLPs) of HPV types 6 and 18 (20 ug each) and 11 and 16 (40 ug each), along with 225 ug of aluminum hydroxyphosphate sulfate as adjuvant and administered as three intramuscular doses at 0, 2 (±1), and 6 (±2) months. The alternate vaccine in market Cervarix, produced by GSK Biologicals, is a bivalent vaccine, which contains VLPs of HPV types 16 and 18 (20 ug each) only with an adjuvant aluminum hydroxide (500 ug) and 3-deacylated monophosphoryl lipid-A (50 ug). Cervarix is also recommended as three intramuscular doses at 0, 1 (up to 2.5 m), and 6 (between 5 and 9 m after 1st dose) months (Table 3.3).

Table 3.3 HPV vaccine characteristics

	Quadrivalent HPV vaccine	Bivalent HPV vaccine
Manufacturer	Merck & Co., Inc.	GlaxoSmithKline
Trade name	GARDASIL® Human Papillomavirus Vaccine Types 6,11,16,18	Cervarix® Human Papillomavirus Vaccine [Types 16, 18] (Recombinant, adjuvanted, adsorbed)
HPV VLPs included	6, 11, 16, 18	16, 18
L1 protein dose	20/40/40/20 μg	20/20 μg
Substrate	*Saccharomyces cerevisiae* (baker's yeast)	Baculovirus expression system
Adjuvant	225 μg of amorphous aluminum hydroxyphosphate sulfate (Merck aluminum adjuvant)	500 μg of aluminum hydroxide and 50 μg of 3-*O*-desacyl-4′-monophosphoryl lipid-A (GSK AS04 adjuvant)

In December 2014, the US Food and Drug Administration (FDA) approved a vaccine called Gardasil-9 to protect females between the ages of 9 and 26 and males between the ages of 9 and 15 against nine strains of HPV. Gardasil-9 is reported to protect against infection with the strains covered by the first generation of Gardasil (HPV6, HPV11, HPV16, and HPV18) along with five other HPV strains responsible for 20 % of cervical cancers – HPV31, HPV33, HPV45, HPV52, and HPV58.

3.9 Therapeutic Vaccines

The HPV vaccines discussed above are both prophylactic and are of no help once the infection is established. Recently several research laboratories have focused on development of therapeutic HPV vaccines, which can efficiently clear off the existing HPV infection. Most of the studies target the HPV oncogenes, E6 and E7, as their expression is critical in HPV persistence and tumorigenesis in the tissue. It is anticipated that targeting these proteins would help eradicate any infection or tumor that might have already been established in the tissue. One such therapeutic HPV vaccine developed by Ricardo Rosales [6] has been clinically tried in Mexico, has gone through clinical trials, and has been approved for use by the Mexican government. It is a recombinant vaccinia viral vaccine MVA E2, composed of modified vaccinia virus Ankara [MVA] expressing the *E2* gene of bovine papillomavirus. In the trial, therapeutic vaccination with MVA E2 proved to be very effective in stimulating the immune system against papillomavirus and in generating regression of flat condyloma lesions in men. Almost 93 % of condyloma patients showed no lesion or presence of papillomavirus as diagnosed by brush histologic examination after 4 weeks of MVA E2 treatment. These patients showed complete elimination of flat condyloma in the urethra and no acetowhite spots were detected over the prepuce. All patients developed antibodies against the MVA E2 vaccine and E2 protein and generated a specific cytotoxic response against papilloma-transformed cells. Viral DNA was not detected in MVA E2-treated patients. Moreover, patients treated with MVA E2 did not show any recurrence of lesions after 1 year of treatment [1].

In another phase I/II clinical trial, the potential use of the MVA E2 recombinant vaccinia virus to treat cervical intraepithelial neoplasia CIN 1, CIN 2, and CIN 3 lesions associated with human papillomavirus (HPV) infection was put to test. Almost 94 % of the patients showed complete elimination of precancerous lesions after treatment with the MVA E2 vaccine. In the remaining 6 %, precancerous lesions were reduced from CIN 3 to CIN 1. All patients developed antibodies against the MVA E2 vaccine, and vaccination generated a specific cytotoxic response against HPV-transformed cells. Furthermore, 50 % of patients showed no evidence of papillomavirus after treatment with MVA E2, while the remaining 50 % showed persistence of HPV DNA but at approximately 10 % of the original viral load. The vaccine has proven to be safe, and as far as is known, there have been no side effects or other documented issues [13].

3.10 Future Perspectives

It is now proven beyond doubt that HPV-associated HNC is a different entity by itself with a better prognosis, survival rates, and favorable outcome. A least invasive and standardized universal testing method for this diagnosis is the need of the hour. Standardization of treatment strategies for HPV-associated HNCs, which can be drastically different from the HPV nonassociated ones, is required to escalate positive outcome. Likewise, more clinical trials and follow-up studies are required to substantiate the efficacy of both quadrivalent and bivalent vaccines in successfully preventing the HPV-associated oral/oropharyngeal cancer. It is still not known whether the therapeutic vaccines made to act against the cervical cancer would prove to be effective in treating the HPV-associated HNCs too and studies should be directed toward answering this.

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Epidemiology and Site-Specific Risk Factors for Oral Cancer

4

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4.1 Introduction and Overview

Most oral malignancies are squamous cell carcinomas arising from the mucous membranes lining the many surfaces of the lip and oral cavity. We define oral cancer as any malignant neoplasm occurring on the lips (both vermillion border and oral aspect), and within the mouth/oral cavity, including all parts of the tongue [ICD 10, C00–C06]. Wherever possible we have excluded the major salivary glands [C07–C08] and nasopharynx [C11] because of their different biology. The oropharynx [C10], pyriform sinus [C12], and hypopharynx [C13] have some commonality in risk factors and behaviour, so data are given for these sites where relevant. Figure 4.1 ranks the global average incidence rates for these head and neck sites.

In the Western world, most oral cancers are diagnosed quite late in their natural history, paradoxically usually without the patient or a clinician being aware of a long-standing pre-existing lesion. This is not to say that long-standing molecular changes were likely present, probably involving a wide area – a 'field of change' – covering much of the upper aerodigestive tract. In contrast, in much of Asia, most oral cancers arise in pre-existing white and red patch, and oral submucous fibrosis.

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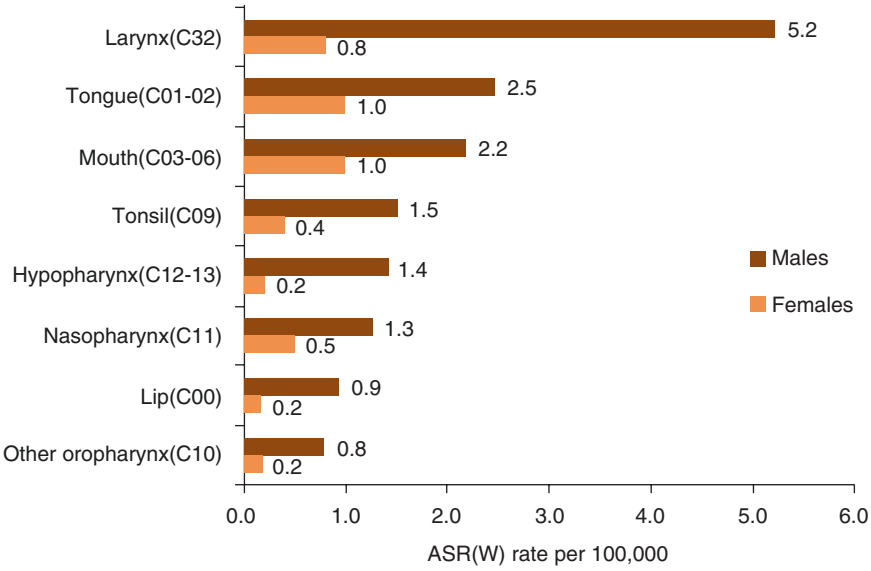


Fig. 4.1 Global results of lip and oral cavity (C00-C06), ASR(W) in comparison to other sites of head and neck cancer by gender and age 0–85+. Age-standardised rates to World Standard Population (*Source*: Cancer Incidence in Five Continents Volume X)

now collectively described as *oral potentially malignant disorders* (*OPMD*) [1]. Brief coverage of the epidemiology of OPMD appears later in this chapter.

Lip oral cavity cancers (ICD 10, C 00- C08) If it is upto 08, it includes salivary gland CA are highly dangerous, accounting for 198,975 cases and 97,919 deaths amongst males and 101,390 cases/47,409 deaths amongst females in the world in 2012 (Table 4.1). The estimated burden of 300,373 cases in 2012 is projected to grow to 450,870 by 2030 (Table 4.2), and whilst this 50 % increase is partly due to population growth and ageing, it is indicative that control of oral cancer is not being achieved. Globally, therefore, 'oral cancer' is the twelfth most common cause of cancer-related mortality amongst males and sixteenth amongst females. However there is marked geographical variation: in Sri Lanka, for example, oral cancer is the leading cancer amongst men, sixth amongst women and second overall. The overall death to registration ratio evident from the above numbers, i.e. D:R = 0.48, is consistent with an average 5-year survival rate of less than 50 %, though cases can do much better if diagnosed early and if strong multidisciplinary teams are responsible for patient care. Sadly, in much of the world, this is not the case. Around the world, with the exception of HPV-related cancers, oral and other head and neck cancers are predominantly diseases of the poor: inequalities and contributing factors are analysed by Johnson et al. [2].

These cancers share common risk factors of tobacco, areca nut and alcohol, with growing evidence for chronic dental trauma and poor oral hygiene, and a role for human papillomavirus: these agents frequently operate in a background of diets poor in antioxidant, vitamins and minerals; inherited polymorphisms in carcinogen-metabolising enzymes play a minor role. There is wide geographical variation in the

Table 4.1 Estimated new cases and deaths in the year 2012, by gender and cancer site for the world, more developed and less developed areas

	Male			Female	
Cancer name	Incidence	Mortality	Cancer name	Incidence	Mortality
Worldwide					
All site cancer[a]	7,427,148	4,653,132	All site cancer[a]	6,663,001	3,547,898
Bladder	330,380	123,043	Bladder	99,413	42,025
Brain, nervous system	139,608	106,379	Brain, nervous system	116,605	83,015
Colorectum	746,298	373,631	Breast	1,676,633	521,817
Gallbladder	76,844	60,334	Cervix uteri	527,624	265,653
Kidney	213,924	90,782	Colorectum	614,304	320,250
Larynx	138,102	73,261	Corpus uteri	319,605	76,155
Leukaemia	200,676	151,317	Gallbladder	101,257	82,479
Lip, oral cavity	198,975	97,919	Kidney	123,936	52,587
Liver	554,369	521,031	Leukaemia	151,289	114,144
Lung	1,241,601	1,098,606	Lip, oral cavity	101,398	47,409
Melanoma of the skin	120,649	31,393	Liver	228,082	224,486
Multiple myeloma	62,469	43,094	Lung	583,100	491,194
Nasopharynx	60,896	35,753	Melanoma of the skin	111,481	24,096
Non-Hodgkin Lymphoma	217,643	115,384	Multiple myeloma	51,782	36,921
Oesophagus	323,008	281,212	Non-Hodgkin Lymphoma	168,098	84,246
Other pharynx	323,008	77,585	Oesophagus	132,776	118,944
Pancreas	178,161	173,812	Ovary	238,719	151,905
Prostate	1,111,689	307,471	Pancreas	159,711	156,560
Stomach	631,293	468,931	Stomach	320,301	254,096
Thyroid	68,179	12,627	Thyroid	229,923	27,142
More developed regions					
All site cancer[a]	3,243,511	1,591,248	All site cancer[a]	2,832,365	1,286,669
Bladder	196,077	58,906	Bladder	57,766	21,016
Brain, nervous system	48,224	36,829	Brain, nervous system	40,743	29,774
Colorectum	398,903	175,389	Breast	793,684	197,528
Gallbladder	27,765	19,003	Cervix uteri	83,078	35,495
Kidney	125,378	47,897	Colorectum	337,964	157,724
Larynx	50,730	22,674	Corpus uteri	167,859	34,715
Leukaemia	80,283	51,314	Gallbladder	34,770	25,830
Lip, oral cavity	68,042	23,380	Kidney	74,613	27,014
Liver	92,018	80,415	Leukaemia	60,991	40,303
Lung	490,267	416,615	Lip, oral cavity	32,781	9,908
Melanoma of the skin	99,379	21,260	Liver	42,284	42,646
Multiple myeloma	36,490	22,335	Lung	267,947	209,830
Nasopharynx	5,071	2,267	Melanoma of the skin	91,687	15,005

(continued)

Table 4.1 (continued)

	Male			Female	
Cancer name	Incidence	Mortality	Cancer name	Incidence	Mortality
Non-Hodgkin Lymphoma	101,873	40,837	Multiple myeloma	31,468	20,690
Oesophagus	67,748	56,094	Non-Hodgkin Lymphoma	88,530	34,251
Other pharynx	44,400	21,420	Oesophagus	18,396	15,241
Pancreas	94,702	93,110	Ovary	99,752	65,892
Prostate	758,739	142,004	Pancreas	92,763	91,300
Stomach	175,117	106,673	Stomach	99,392	68,037
Thyroid	29,672	3,651	Thyroid	93,104	6,740
Less developed regions					
All site cancer[a]	4,183,637	3,061,884	All site cancer[a]	3,830,636	2,261,229
Bladder	134,303	64,137	Bladder	41,647	21,009
Brain, nervous system	9,184	69,550	Brain, nervous system	75,862	53,241
Colorectum	347,395	198,242	Breast	882,949	324,289
Gallbladder	49,079	41,331	Cervix uteri	444,546	230,158
Kidney	88,546	42,885	Colorectum	276,340	162,526
Larynx	87,372	50,587	Corpus uteri	151,746	41,440
Leukaemia	120,393	100,003	Gallbladder	66,487	56,649
Lip, oral cavity	130,933	74,539	Kidney	49,323	25,573
Liver	462,351	440,616	Leukaemia	90,298	73,841
Lung	751,334	681,991	Lip, oral cavity	68,617	37,501
Melanoma of the skin	21,270	10,133	Liver	185,798	181,840
Multiple myeloma	25,979	20,759	Lung	315,153	281,364
Nasopharynx	55,825	33,486	Melanoma of the skin	19,794	9,091
Non-Hodgkin Lymphoma	115,770	74,547	Multiple myeloma	20,314	16,231
Oesophagus	255,260	225,118	Non-Hodgkin Lymphoma	79,568	49,995
Other pharynx	70,731	56,165	Oesophagus	114,380	103,703
Pancreas	83,459	80,702	Ovary	138,967	86,013
Prostate	352,950	165,467	Pancreas	66,948	65,260
Stomach	456,176	362,258	Stomach	220,909	186,059
Thyroid	38,507	8,142	Thyroid	136,819	20,402

Source: GLOBOCAN, 2012

[a]Excludes non-melanoma skin cancer

incidence of cancer arising at sub-sites of the oral cavity and pharynx, depending on the dominant behavioural risk factors in particular cultures. For example, betel quid chewing affects buccal and retro-molar sites, alcohol is related to the tongue and floor of the mouth and sexually transmitted HPV-related cancers are found mostly in the tonsil and base of the tongue.

Table 4.1 shows the number of new cases and the number of deaths from lip plus oral cancer estimated around the world in 2012 (the latest international data available at the time of going to press), along with all other cancer sites for comparison. Table 4.2 gives the projections for oral cancer in more detail.

Table 4.2 Projected burden of oral cancer incidence by age and gender for the world, more developed and less developed areas by year, 2012 and 2030

Lip and oral cavity (C00-C06)					
	Year	Estimated number of new cancers (all ages)	Male	Female	Both sexes
World	2012		198,975	101,398	300,373
		Ages < 65	128,866	56,401	185,267
		Ages ≥ 65	70,109	44,997	115,106
	2030		298,854	152,016	450,870
		Ages < 65	172,377	74,412	246,789
		Ages ≥ 65	126,477	77,604	204,081
More developed countries	2012		68,042	32,781	100,823
		Ages < 65	38,559	137,50	52,309
		Ages ≥ 65	29,483	19,031	48,514
	2030		82,780	40,033	122,813
		Ages < 65	39,275	13,604	52,879
		Ages ≥ 65	43,505	26,429	69,934
Less developed countries	2012		130,933	68,617	199,550
		Ages < 65	90,307	42,651	132,958
		Ages ≥ 65	40,626	25,966	66,592
	2030		208,833	111,358	320,191
		Ages < 65	128,478	60,642	189,120
		Ages ≥ 65	80,355	50,716	131,071

Source: GLOBOCAN
Population forecasts were extracted from the United Nations, World Population prospects, the 2012 version. Numbers are computed using age-specific rates and corresponding populations for 10 age groups

Table 4.3 Oral cancer incidence by race/ethnicity expressed, cases per 100,000 for the USA

Gender	All races	White	Black	American Indian/Alaska Native	Asian or Pacific Islander	Hispanic	White Hispanic	White non-Hispanic
Male	15.5	15.7	17.2	9.7	10.9	9.2	9.3	16.7
Female	6.1	6.1	5.7	5.3	5.5	3.6	3.7	6.5

Source: Surveillance, Epidemiology End Result Programme (http://seer.cancer.gov/)
SEER cancer registries use the 2000 US standard population based on single years of age from the Census P25-1130 series estimates of the 2000 US population

4.2 Cancer Registries

Cancer registries play a vital role in monitoring the incidence of, and mortality from, cancers. However, the quality of data available is highly variable. Many parts of the world produce no data at all; in others (often amongst the most populous), the data may come from localised, atypical regions. Hospital-based cancer registries naturally gather biased information – those cases which present to hospital only; thus, in many developing countries, cases may not come to attention at all, either because of fear or the inability of poor people to access hospital services. Data may be even more unreliable because, in many resource-poor countries, follow-up, even of treated cases, is impossible. Death certification is not always compulsory, and there is limited international standardisation in the categories for cause of death, let alone calibration of those signing death certificates.

Fortunately, many nations have high quality national, often incorporating regional, population-based cancer registries, with compulsory reporting of all malignancies. These are guided by, and quality assured by, both national authorities and the positive influence of the World Health Organisation (WHO), mostly through its constituent body, the International Agency for Research on Cancer headquartered in Lyon, France. Data from all over the world are collated and are available from the websites of both these bodies: this includes free access to programmes that allow on-line interrogation of the databases. Many of the tables and graphs in this chapter have been generated in this way. Within the USA, the SEER website provides similar sophisticated opportunities to registered users (SEER is the Surveillance, Epidemiology and End Results programme of the National Cancer Institute). It is based on data from, nowadays, 20 population-based registries, but these by no means cover the whole nation. Some outputs are based on a subset of these registries. See http://seer.cancer.gov/registries/list.html.

4.3 Why Collect Detailed Epidemiological Data?

Cancer epidemiology is a demanding but essential science. Some acquaintance with epidemiological method and data is required by all who participate in cancer care, from politicians, public health officials, hospital managers, individual clinicians in both general and the wide range of specialist practitioners concerned with diagnosis and treatment, those providing palliative care, nurses, speech and swallowing therapists, dieticians and social workers to spiritual advisors. *Descriptive epidemiology* provides the fundamental evidence base, but its value is dependent on the accuracy and completeness of the information therein: reliable, sufficiently detailed and safely stored hospital-based information is *sine qua non*. Increasingly, hospital records contain information on lifestyle and other known or suspected risk factors. The growth of biological ‘tumour banks’ or ‘tissue banks’ from which molecular markers and indeed molecular mechanisms can be researched is encouraging: this needs co-ordinated international action. There are several large, often international consortia, using such banks to unravel the genome of all cancers: notably the

International Cancer Genome Project which has several collaborating centres dealing with head and neck (https://icgc.org/), the Cancer Genome Atlas in the USA (http://www.genome.gov/17516564) and the Wellcome Trust Sanger Institute Cancer Genome Project in the UK (https://www.sanger.ac.uk/research/projects/cancergenome/).

Population-based registries, as described above, are of even greater value. These permit *analytical epidemiology* and thus the ability to address essential questions such as: Why is the incidence of a particular type or site of neoplasm rising or falling over time or in a particular ethnic group or age group? How should this inform government and public health policy? Are existing public awareness and screening campaigns effective and efficient? How do different treatment modalities compare? How does my hospital or my personal clinical practice compare to the national average or world best practice? In respect of the latter, there is an ethical imperative for every clinician to keep detailed records, using standardised measures, of the outcomes of his or her care. Guidelines for Care Pathways and 'Minimum Data-Sets' to facilitate quality control and recording of outcomes are available: those from the British Association of Head and Neck Oncologists (http://www.bahno.org.uk/docs/) and from the American Head and Neck Society (http://www.headandneckcancer.org/) can be recommended. In many countries, cancer is a notifiable disease and both the registration of all cases and the provision of information on the patient, on the care provided and on the outcomes – not just survival rates but information on complications and on quality-of-life measures – are mandatory. The guidelines from the National Comprehensive Cancer Network in the USA are invaluable (http://www.nccn.org/professionals/physician_gls/f_guidelines.asp#site). There remains, however, a continuous need to evaluate the quality and strength of the evidence base for all published guidelines, preferably using the strict criteria of the GRADE approach (grading of recommendation, assessment, development and evaluation: http://www.gradeworkinggroup.org/intro.htm).

4.3.1 Geographical Epidemiology

Those parts of the world with the major disease burden are readily grasped from the World Mapper image in Fig. 4.2. This website produces maps in which the area of the globe occupied by a country is distorted to represent the volume of the data presented. Clearly most deaths occur in the Indian subcontinent and neighbouring countries.

Disease burden is a product of incidence rate and population size. Variations in incidence and mortality around the world are further illustrated in the bar charts (Fig. 4.3a, b) and in the series of maps derived from the Globocan website (Fig. 4.4a–d). Examined on-line, these maps are 'live' and details for individual countries immediately apparent. More than half of the lip and oral cavity cancer cases and nearly 66.3 % of cancer deaths are seen in Asia followed by Europe (18.4 %), Africa (6.1 %), Latin America and Caribbean (5.1 %) and Northern America (3.1 %). The highest crude rates in the world are found in Melanesia,

Maldives, Sri Lanka, Bangladesh, France and Hungary. In India alone over 100,000 cases of oral cancer are registered every year and the numbers are rising. Though men predominate overall, amongst females, a very high incidence is found throughout South Central Asia (4.7 per 100,000 pa) [3].

Melanesia shows the highest age-standardised incidence rates [ASR(W)] in the world: for males (22.9 per 100,000 pa) and females (16.0 per 100,000 pa) [3]. The extremely high rates in the relatively small populations of the Melanesian Islands have not been comprehensively researched, but data from Papua New Guinea (PNG) define the importance of areca nut (betel) chewing (called buai in PNG) and smoking habits as the major risk factors. Data for Africa are not particularly robust.

More than 126,000 cases of oral cancer (ICD 10, C00–C06) occur every year in South and South-East Asia alone, with poor prospect of survival: about 90 % of these cases are attributable to smoking and chewing habits. The serious situation in India is clear from Figs. 4.5 and 4.6. It is encouraging that overall rates in India are showing a decreasing trend in successive birth cohorts, declining trends being observed for mouth (ICD 10, C03–C06) and tongue (C01–C02) cancers amongst females and tongue cancers amongst males between 1982 and 2000 [4], and this has continued. However, population growth in the subcontinent means that the disease burden continues to rise: better primary prevention is essential (Fig. 4.5) [5]. There is serious concern that widespread use of commercial areca nut and tobacco

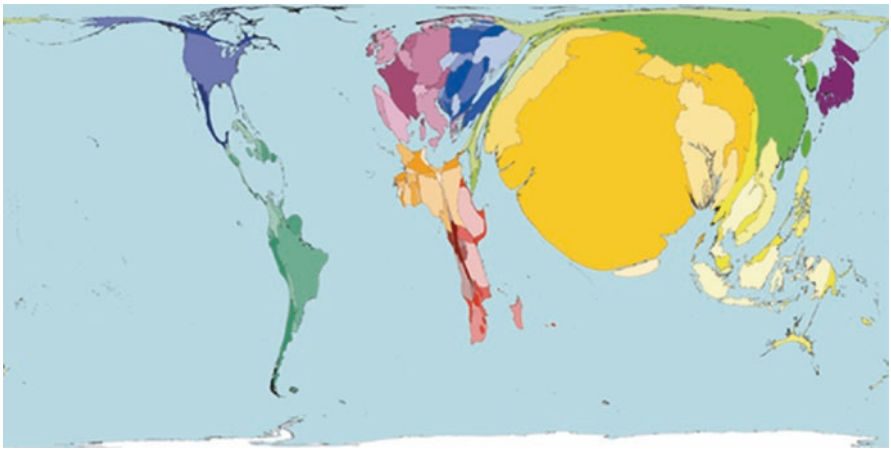
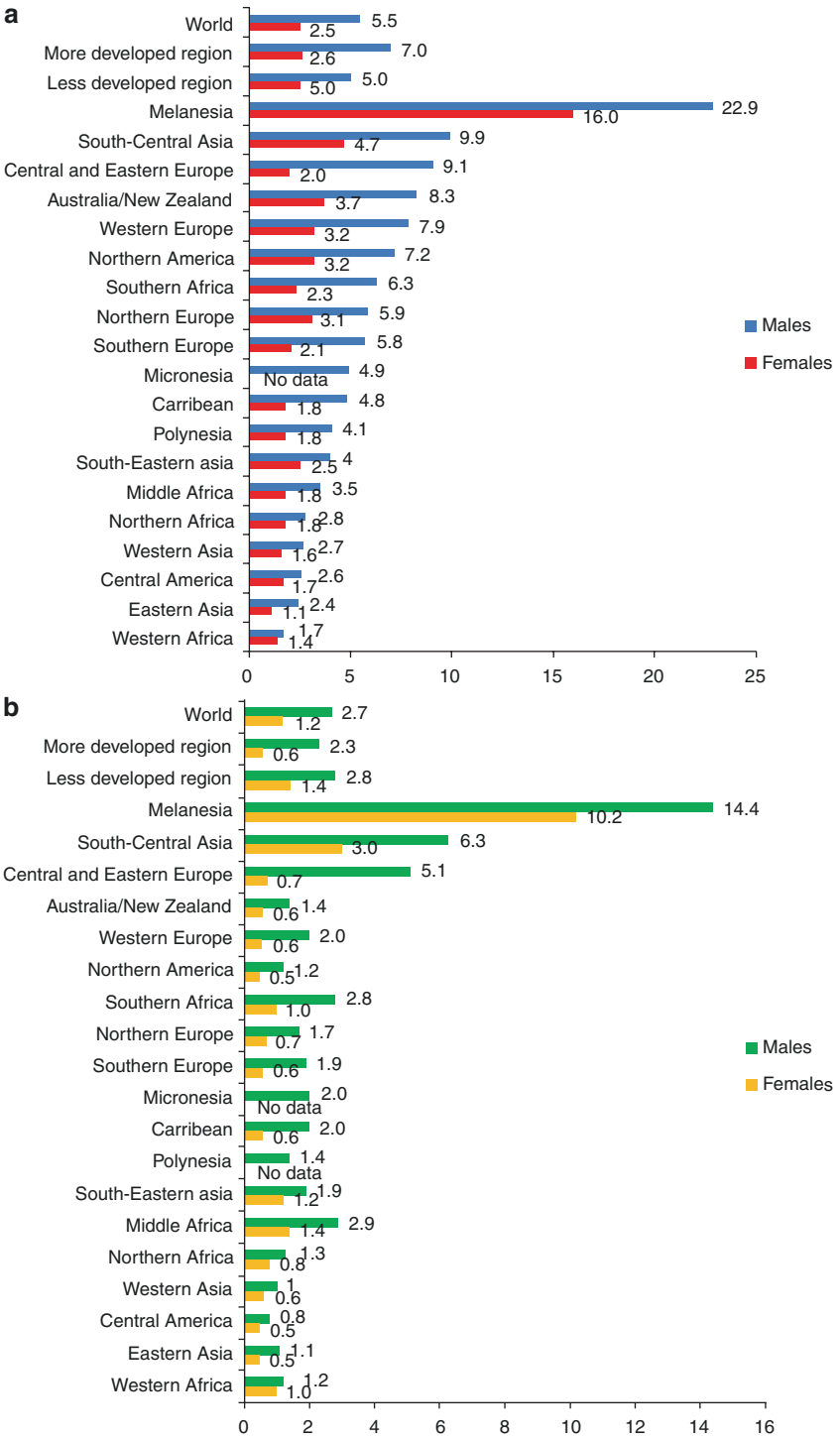


Fig. 4.2 Number of deaths from cancer of the mouth and pharynx (C00-C14), males only (Worldmapper gives the data in the year 2002. Available at http://www.worldmapper.org/display_extra.php?selected=419. Accessed on 10-02-2015)

Fig. 4.3 (**a**) Geographical variation in incidence rates expressed in ASR(W) of the lip plus oral cavity (C00-C06) per 100,000 pa for males and females, all ages. (**b**) Geographical variation in mortality rates expressed in ASR(W) of the lip plus oral cavity per 100,000 pa for males and females, all ages (*Source:* GLOBOCAN, 2012)


a
World
More developed region
Less developed region
Melanesia
South-Central Asia
Central and Eastern Europe
Australia/New Zealand
Western Europe
Northern America
Southern Africa
Northern Europe
Southern Europe
Micronesia
Carribean
Polynesia
South-Eastern asia
Middle Africa
Northern Africa
Western Asia
Central America
Eastern Asia
Western Africa
5.5
2.5
7.0
2.6
5.0
5.0
22.9
16.0
9.9
4.7
9.1
2.0
8.3
3.7
7.9
3.2
7.2
3.2
6.3
2.3
5.9
3.1
5.8
2.1
4.9
No data
4.8
1.8
4.1
1.8
4
2.5
3.5
1.8
2.8
1.8
2.7
1.6
2.6
1.7
2.4
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1.4
0
5
10
15
20
25
Males
Females
b
World
More developed region
Less developed region
Melanesia
South-Central Asia
Central and Eastern Europe
Australia/New Zealand
Western Europe
Northern America
Southern Africa
Northern Europe
Southern Europe
Micronesia
Carribean
Polynesia
South-Eastern asia
Middle Africa
Northern Africa
Western Asia
Central America
Eastern Asia
Western Africa
2.7
1.2
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6.3
3.0
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0.7
1.4
0.6
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1.2
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0.6
2.0
No data
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0.6
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0.8
1
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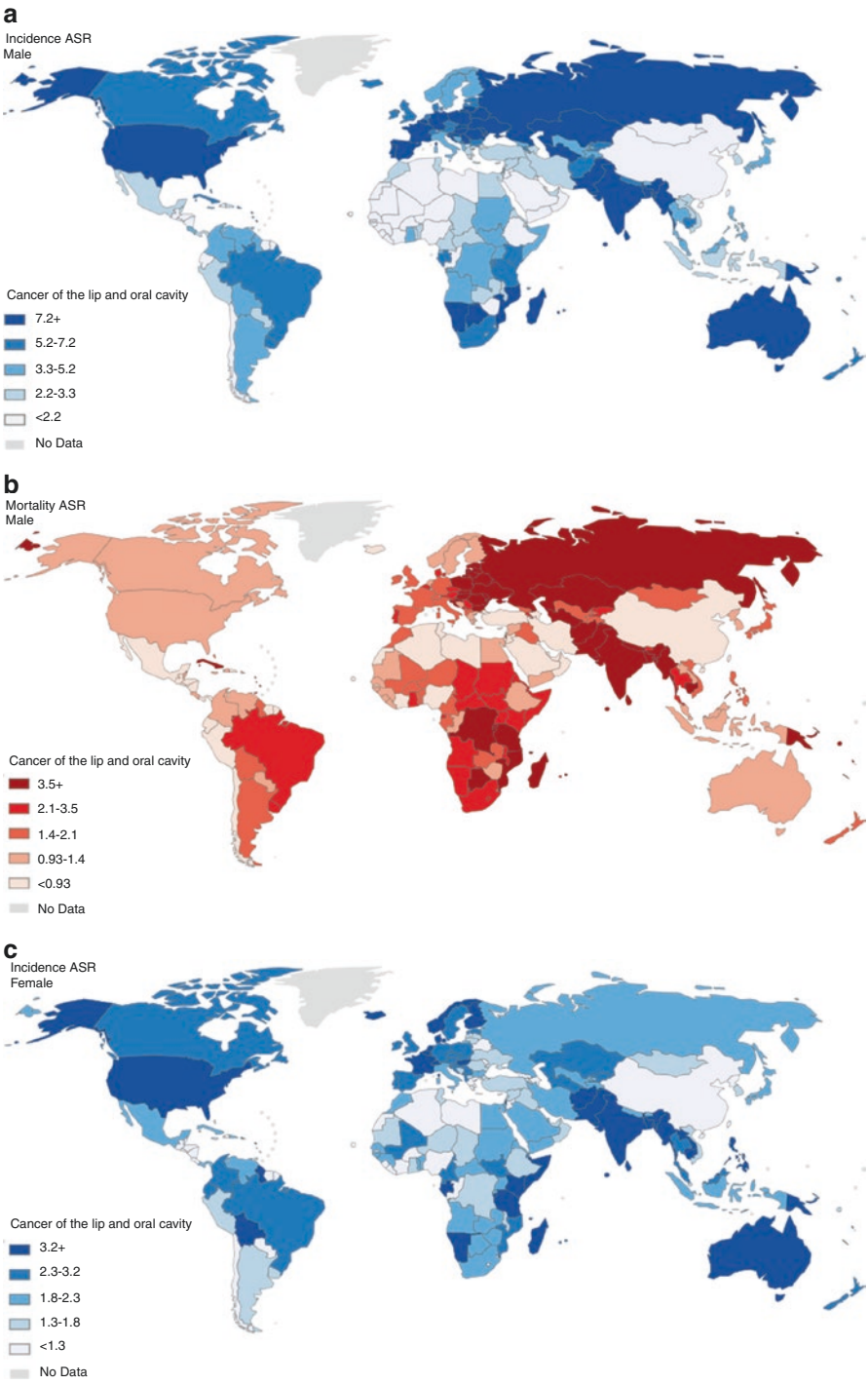
2
4
6
8
10
12
14
16
Males
Females


a
Incidence ASR
Male
Cancer of the lip and oral cavity
7.2+
5.2-7.2
3.3-5.2
2.2-3.3
<2.2
No Data
b
Mortality ASR
Male
Cancer of the lip and oral cavity
3.5+
2.1-3.5
1.4-2.1
0.93-1.4
<0.93
No Data
c
Incidence ASR
Female
Cancer of the lip and oral cavity
3.2+
2.3-3.2
1.8-2.3
1.3-1.8
<1.3
No Data

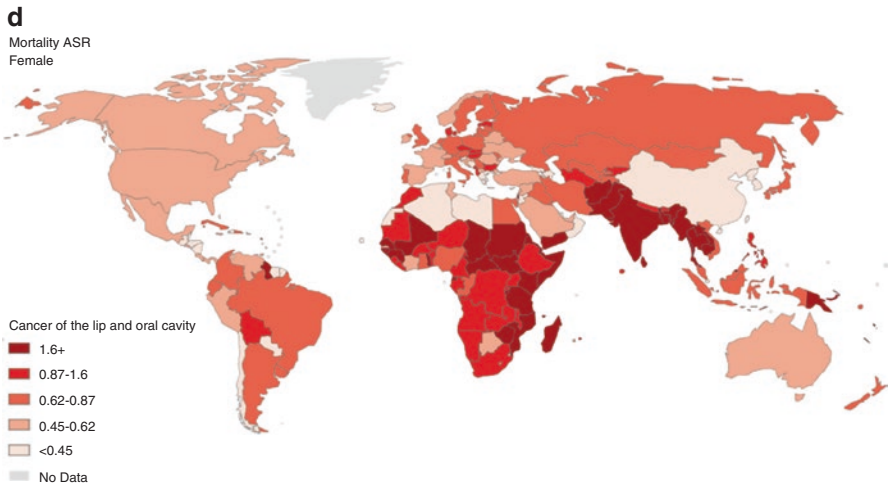


Fig. 4.4 (continued)

Fig. 4.4 (**a**–**d**) Geographical variation in incidence and mortality rates expressed in ASR(W) for the lip plus oral cavity (C00-C06) per 100,000 pa for males and females, all ages. Data derived from the Globocan 2012 database. (**a**) Estimated age-standardised incidence rates of the lip plus oral cavity (C00-C06) amongst males, worldwide in 2012. (**b**) Estimated age-standardised mortality rates of the lip plus oral cavity (C00–C06) amongst males worldwide in 2012. (**a**, **b**) Incidence (**a**) and mortality (**b**) rates for the lip and oral cavity cancer in males, in quintiles, by country. A quick comparison of these maps makes a number of points. The 'traditional' high incidence areas of Central Asia and the Indian subcontinent stand out: much of this is due to betel quid use, with or without smokeless tobacco, plus smoking, sometimes alcohol abuse, and poor diet. Note that parts of both Western and Eastern Europe remain in the top quintile. The African data are not particularly robust. Australia shows a high incidence, due to ultraviolet light-induced lip cancer in a fair-skinned population: mortality rates are not comparably high because lip cancer is comparatively easily treated. Eastern Europe and the former Soviet Republics have high mortality, partly related to low socio-economic status, limited treatment facilities and the fact that many patients have substantial co-morbidities. As stated in the text, Papua New Guinea and surrounding Melanesian islands of the Western Pacific are in the top quintile both in incidence and mortality: Melanesia has the highest recorded rates in the world – associated with chewing of areca nut and tobacco use. (**c**) Estimated age-standardised incidence rates of the lip plus oral cavity (C00–C06) amongst females worldwide in 2012. (**d**) Estimated age-standardised mortality rates of the lip plus oral cavity (C00–C06) amongst females worldwide in 2012. (**c**, **d**) Similar explanations relate to the national incidence (**a**) and mortality (**b**) data for women for cancers of the lip and oral cavity. Note the serious situation in the Indian subcontinent, much of Northern Asia, South America and parts of the Middle East including the southern provinces of Saudi Arabia and Yemen. In parts of India, oral cancer is the leading cancer amongst women, because of heavy use of betel quids. Indeed emigrant Tamil women working on rubber and palm oil estates in Malaysia have amongst the highest rates, by population group, in the world

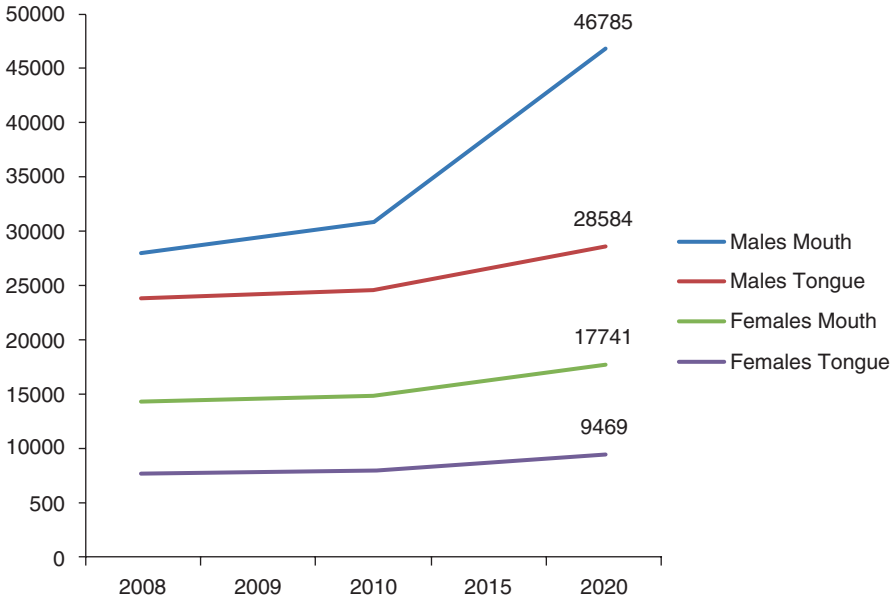


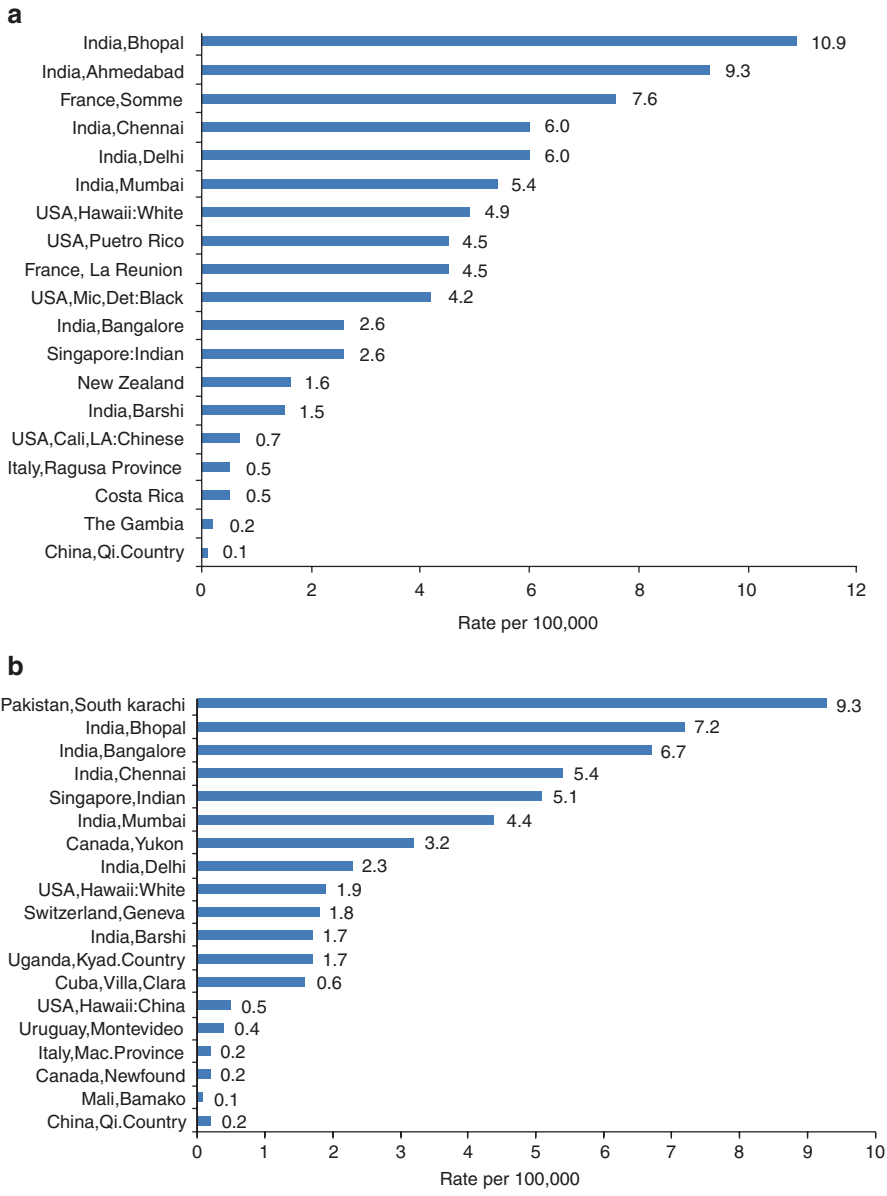
Fig. 4.5 Projected rises in the burden of mouth and tongue cancer in India in the next decade, mouth C03–06, tongue C01–02 [5] (*Source*: Derived from ICMR Report Chapter 5: Projection of Burden of Cancer)

products has created rises in the incidence of oral submucous fibrosis and of subsequent oral cancer, first noted nearly two decades ago [6].

Data from Japan show a dramatic increase in oral and pharyngeal cancer incidence (ICD 10, C01–C14) for both sexes; there was a 4.4-fold increase for males and 3.8-fold increase for females in the total numbers between 1965 and 1999 – noted from data retrieved from the Osaka Cancer Registry [7]. There is also an upward trend for both males and females in Australia for the oropharynx

Fig. 4.6 (**a**) International comparisons of age-adjusted incidence rates from the population-based cancer registries of the Indian National Cancer Registry programme: TONGUE ICD-10: C01-C02 – Males, 2001–2002 (*Source*: Derived from 'Development of an Atlas of Cancer in India': Indian Council for Medical Research. Available at: http://www.canceratlasindia.org/chapter6_Report.aspx?SiteName=Mout&ReportType=Int_Graph&Sex=M&MyBtn=View+Graph). (**b**) International comparisons of age-adjusted incidence rates from the population-based cancer registries of the Indian National Cancer Registry programme: MOUTH (ICD-10: C03-C06) – Female, 2001–2002 (PBCR: Population based cancer registry. Data derived from: http://www.canceratlasindia.org/chapter6_Report.aspx?SiteName=Mout&ReportType=Int_Graph&Sex=F&MyBtn=View+Graph). (**a**, **b**) Note the considerable differences between the Indian population-based registries which, nevertheless, are higher than most of the other countries shown here. The Karachi population has similar habits to those of much of India, namely, heavy use of smokeless tobacco in various forms, and of areca nut chewing, synergised by poor diet in the lower socio-economic groups. Females are often the heaviest chewers, as smoking is not socially acceptable amongst women

(Ariyawardana A and Johnson 2013) and amongst the non-Maori population in New Zealand. Lip cancer in fair-skinned populations, particularly due to ultraviolet light, is a serious problem [8]. In Europe, Hungary has the highest incidence and mortality of oral and pharyngeal cancer for both sexes [9]. Between 1984 and 1994, the Hungarian mortality rates for oral cancers rose by 83.5 and 72.3 % in males and females, respectively, but this has now stabilised. Trends in the mortality rate



amongst Italian and French males peaked in the 1980s and have decreased after 1990 [10]. However, persisting upward trends were registered for Belgium, Denmark, Greece, Portugal and Scotland around the turn of the millennium [11].

In the USA, the estimated number of incident cancer cases for tongue, mouth and other oral cavities in 2008 was 15,250 cases for men and 7,650 for women. In the USA, the mortality rates per 100,000 population pa for cancer of the oral cavity and pharynx for men were 5.61 in 1990 and 3.98 in 2004, the absolute decrease being 1.63 per 100,000, contributing to a 3 % reduction in mortality of all sites. For women, the decrease across the same period was 0.56, contributing to a 2.5 % reduction of all sites [7]. The incidence rates of cancers of the oral cavity were stable or declining for men and women in most age groups during the period 1973–2003 in the USA, probably related to changes in tobacco and alcohol consumption. These trends have continued (Table 4.4). This is a highly pleasing situation, common to many countries with advanced care facilities but not reflected in most of the high incidence countries elsewhere in the world. Furthermore, Black citizens of the USA fare comparatively badly.

4.3.2 Differences by Sex

As already noted, worldwide, the incidence of oral cancer is higher for males than females [3]. Overall the age-specific incidence of 'oral cavity' cancers was 5.5, per 100,000 population pa for males in 2012 and 2.5 for females. Within Europe, Spain (8.6 per 100,000) after Tasmania (7.6 per 100,000) shows the highest ASR(W) amongst males. Amongst females, Tasmania (2.0 per 100,000) and Western Australia (1.8 per 100,000) show very high ASR(W) for cancer of the lip. Male predominance is related to their greater indulgence in the most important risk factors, heavy alcohol and tobacco consumption for intra-oral cancer and sunlight for lip cancer, especially those who work outdoors.

Bhopal City from India shows the highest ASR(W) for cancer of the tongue (10.3 per 100,000) and mouth (9.6 per 100,000) amongst males globally [3, 12]. Bhopal is followed by Hawaii for cancer of the tongue amongst males (6.4 per 100,000). Similarly, amongst females in Australia, Northern Territory, indigenous people show the highest ASW(R) for cancer of the mouth (5.2 per 100,000), but the numbers are small [3].

After Bhopal from India, the highest ASR(W) for cancer of the mouth amongst males is seen in France followed by Brazil: population-based cancer registries from

Table 4.4 Mortality trends (annual percentage change) for oral and pharyngeal cancer in the USA between 2002 and 2011, by race and sex. (SEER Cancer Statistics Review, 1975–2011, published in 2014) [22] (Howlader et al. 1975–2011)

	All races			Whites			Blacks		
	Total	Males	Females	Total	Males	Females	Total	Males	Females
All ages	−1.0[a]	−0.9[a]	−1.5[a]	−0.6[a]	−0.4[a]	−1.3[a]	−3.4[a]	−3.7[a]	−2.7[a]

[a]Indicates that the annual percentage change in rate is statistically significantly different from zero ($p < 0.05$)

Sao Paulo and Puerto Alegre have registered very high rates of cancer of the tongue and of mouth [13].

In the recent past oral cancer in females has increased in some parts of the world. For instance, a study from Argentina showed the male/female ratio to be 1.24:1 for the period 1992–2000 compared to 7.1:1 for the 1950–1970 period [14]. The incidence of tongue and other intra-oral cancers for women can be greater than or equal to that for men in high incidence areas such as India, where betel quid/areca nut chewing (and sometimes smoking) is common amongst women – although this varies considerably from region to region. For example, many cities from India like Bangalore (6.3 per 100,000 pa), Bhopal and Chennai show the highest ASR(W) for cancer of the mouth amongst females in the world [5, 12]. Similarly, after Asia, women as compared to males from Western Australia show high incidence rates for cancer of the mouth which may be due to increasing prevalence of smoking habits and exposure to sunlight amongst them as shown in Fig. 4.3 [15].

Early this century, within Europe, the incidence of oral cavity and pharyngeal cancers (C00–C14) amongst males varied substantially between 5.9 (Finland) and 32 (France) per 100,000 pa [16]. Incidence rates amongst females were highest in Northern and Western Europe but were consistently lower than those for males. The male-to-female ratio decreased during the last 10 years and recently varied between 1.5 and 2.5 in much of Northern Europe, but with 7.7 in Lithuania. Between 1990 and 1999, the UK incidence rates for oral cancers rose in males of all ages from 6.5 to 8.3 per 100,000 (an increase of 18 %) and in females from 2.6 to 3.6 per 100,000 (an increase of 30 %), and this continues to be a concern [17].

The most common risk factors for cancers of the lip and oral cavity are smoking tobacco, the use of smokeless tobacco, alcohol and HPV infections with smoking and alcohol use having synergistic and cumulative effects [18]. Indulgence in these habits differs between the sexes according to socio-cultural norms. Smokeless tobaccos (most commonly used as paan with and without tobacco) in its all forms are major risk factors in South Asia and the Pacific indulged in by both men and women, even children [19]. Betel quid without tobacco is very popular in Taiwan and China amongst men and amongst both men and women in Papua New Guinea. Men in Taiwan who chew betel quid without tobacco are 24 times at a greater risk of developing cancer of the lip and oral cavity than those who do not chew. Apart from the traditional risk factors, it has been suggested that oestrogen deficiency may influence susceptibility to oral cancer in women: significantly, younger mean age at menopause and higher rates of hysterectomy may influence the higher rates of oral cancer seen amongst younger females [20]. Thus, data presented in this chapter are, whenever possible, separated by sex.

4.3.3 Differences by Race/Ethnicity

Variations by ethnicity are largely due to social and cultural practices, social inequalities and the influence of dietary and genetic factors, though the latter are poorly quantified by racial group. Variations in outcome are contributed to by

differences in access to health care. Where cultural practices represent risk factors, their continuation by emigrants from high incidence regions to other parts of the world results in comparatively high cancer incidence in immigrant communities. This can also affect the sub-sites of oral cancer most commonly effected, as shown in a study from California [21]. In the USA the highest rates for head and neck sites overall, including the lip and mouth, are found amongst Black men and non-Hispanic White men followed by non-Hispanic women with Asian and Hispanic populations showing lower incidence rates. Tongue cancer was the most common type of oral cancer amongst every ethnicity. Asians were more likely to develop their malignancy in the buccal mucosa, a reflection of continuing areca and tobacco chewing habits in some cultures. Another study showed that American Indians and Alaskan Natives overall had significantly lower incidence rates than non-Hispanic Whites [22]. Several studies from the USA have demonstrated that Black patients with oral cancer have poorer overall and disease-specific survival than Whites, mainly because of their comparatively poor access to health care [23, 24]. This is especially concerning because the incidence of oral plus pharyngeal cancer for Black men in the USA is so high and is the sixth most common site for malignant disease amongst this group [25].

In the Republic of South Africa, amongst Asian/Indian South Africans, oral and oropharyngeal cancer incidence was higher amongst females (ASIR = 4.60) than amongst males (ASIR = 3.80). Excluding those involving the lip, these cancers were highest amongst Coloureds (ASIR = 5.72) and lowest amongst Blacks (ASIR = 3.16). Incidence rates increased significantly amongst Coloured South Africans over the period from 1992 to 2001 ($p < 0.05$), particularly for the oropharynx [26].

The age-adjusted incidence rate for oral cancer is higher for South Asians than for other residents in England, particularly amongst females [27]. Interestingly, this study showed that British South Asian males have significantly better survival than their non-South Asian peers in the southeast of England, possibly a reflection of the more indolent progress of tobacco/areca nut-induced lesions [27].

4.4 Age Distributions

Oral cancer is usually a disease that occurs in males after the fifth decade of life. The mean age at presentation is in the fifth and early sixth decades in Asian populations compared with the seventh and eighth decades in the North American population [28–33]. Statistics in the USA for 1975–2011 show that the median age at diagnosis for cancer of the oral cavity was 62 years [34].

Several studies suggest that 4–6 % of oral cancers now occur at ages younger than 40 years [35]. An alarming increase in incidence of oral cancers amongst younger people has been reported over the past few decades from many parts of the world [36–38], a trend that appears to be continuing. There was a significant increase in the incidence of cancers in the tongue and tonsil amongst 20–40 year olds in the USA between 1973 and 2000 [39]. In Germany, Czechoslovakia and

Hungary, there has been an almost tenfold rise in mortality from oral cancer in men aged 35–44 [40], within one generation. Robinson and Macfarlane showed a dramatic increase in incidence rates for younger males in Scotland from the 1980s to the 1990s [41]. In the high prevalence areas of the world, in many cases patients are less than 40 years old, probably owing to heavy use of various forms of tobacco from an early age, although some recent Indian data have not shown this [42].

It is also clear that a number of cases of squamous cell carcinoma occur in both young and old patients in the absence of traditional risk factors, and in which the disease may pursue a particular aggressive course, more so in the elderly. A study conducted in Southern England concluded that a substantial proportion of cases of younger people diagnosed with oral cancer occur in the absence of known risk factors [35]. This, together with the relatively short duration of exposure in users, suggests that factors other than tobacco and alcohol are implicated in the development of oral cancer in a significant minority of cases. Diets poor in fresh fruits and vegetables were identified as conferring significant risk. There is now substantial evidence that human papillomavirus infections are driving this rise in younger adults, but, fortunately, HPV-related oropharyngeal cancers respond well to radiotherapy, permitting treatment de-escalation and improved quality of life. It is also suggested that greater attention should be paid to familial antecedents of malignant neoplasms in younger patients with oral cancer [43].

Age distribution curves for oral cancer – for the lip and mouth separately and for males and females separately – are given for selected countries in Fig. 4.7a–d. See legends for further interpretation.

4.5 Mortality Rates and Trends over Time

Trends of age-standardised (world population) mortality rates for the lip, oral cavity and pharynx combined, within selected countries over the past three to six decades, are presented in Fig. 4.8a, b [44]. Such trends in mortality over time are important to track and to understand. See legends for further interpretation.

There was a steady rise in oral cancer mortality in men from the 1950s to late 1980s in most Western European countries [45], but this trend has since declined, e.g. in France, which had exceedingly high rates in the past. Unfortunately, in most countries in Central and Eastern Europe, oral cancer mortality in men continued to rise, reaching exceedingly high rates in Hungary, Slovakia, Slovenia and the Russian Federation at the end of the last century. Hungary, Ukraine, Estonia and Bulgaria showed more than a 100 % increase in mortality rates for men during the 20-year period up to the turn of the millennium. Even though the rates of oral cancer are comparatively low amongst women, there was an increase in several countries in Europe (notably Hungary, Belgium, Denmark and Slovakia) over this period. These disturbing rises are thought to have been related to high drinking and smoking patterns in these societies, together with poor diet in lower socio-economic groups. Fortunately improvements are now evident.

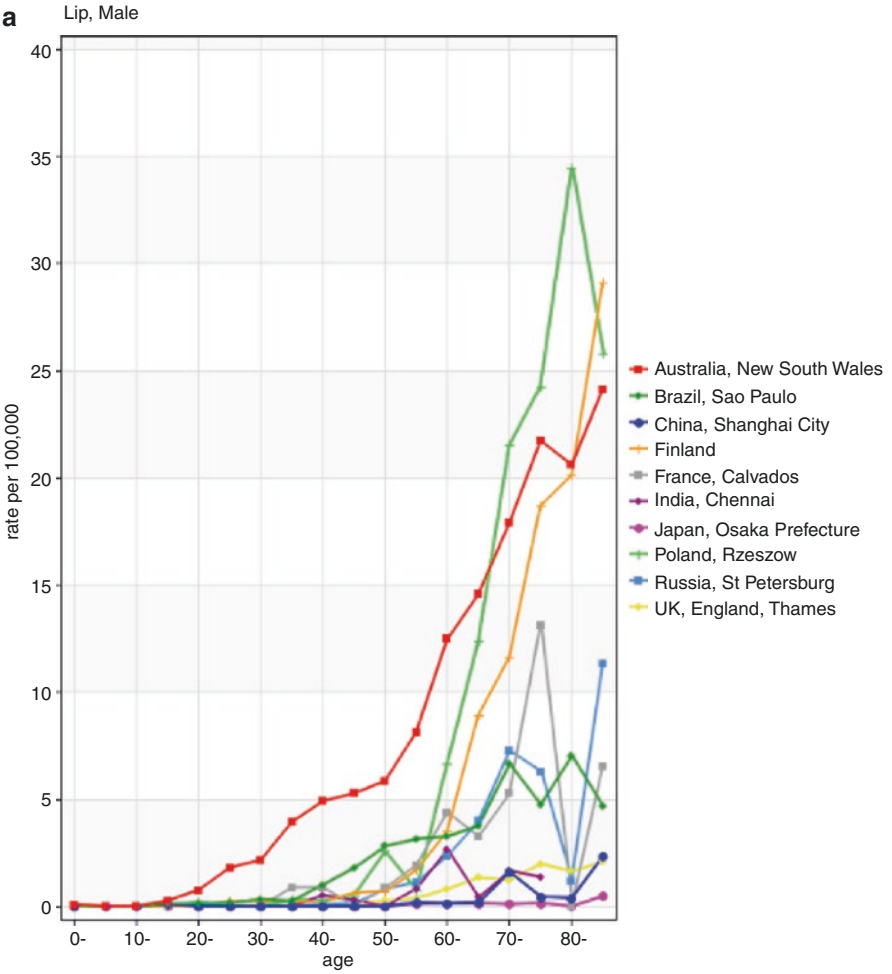
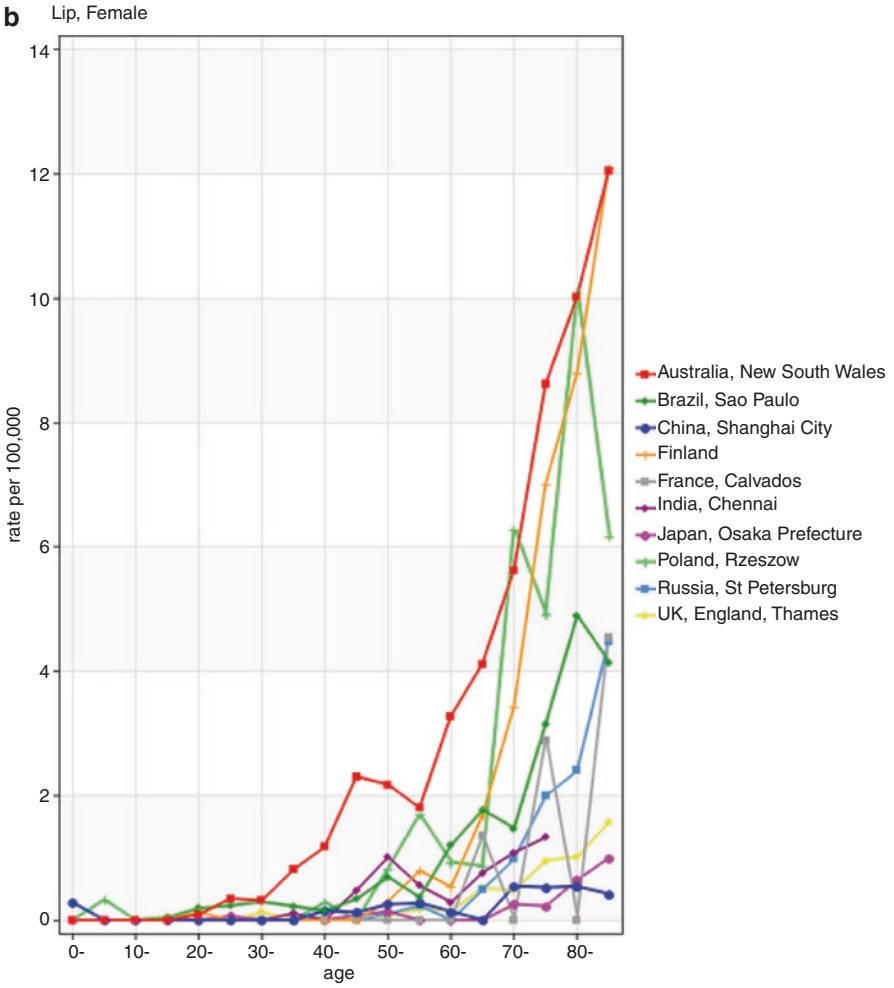


Fig. 4.7 (**a**) Male age-specific incidence curves for the lip (C00) for selected countries. Most cases occur in the sixth to seventh decades of life, presumably because decades of exposure to tobacco, alcohol and poor nutrition take time to synergise with other agents in triggering malignant transformation – or in allowing this to survive the host response! There are disturbingly high rates of cancer of the lip in Poland, Finland and Australia in the later stages of life. What is surprising are the low rates recorded for Shanghai, in spite of high smoking prevalence in this large city. China is currently developing a more comprehensive, nationwide cancer registry system so more cogent data will soon be available. (**b**) Female age-specific incidence curves for the lip (C00) for selected countries. There is an alarming increase in rates of oral cancer amongst women from Australia, Finland and Russia. (**c**) Male age-specific incidence curves for the mouth (C03–C06) for selected countries. The trends from cancer of the mouth being diagnosed in later stages of life are shifting to mid-stage of life in many countries like France, India, Russia and Poland, followed closely by Brazil. However in Japan and China, most of the cases are still diagnosed in later stages of life. (**d**) Female age-specific incidence curves for the mouth (C03–C06) for selected countries. Apart from the significant early onset amongst women in South India – due to areca nut and tobacco chewing – mouth cancers amongst females over much of the world show similar age distribution and are diagnosed in later stages of life



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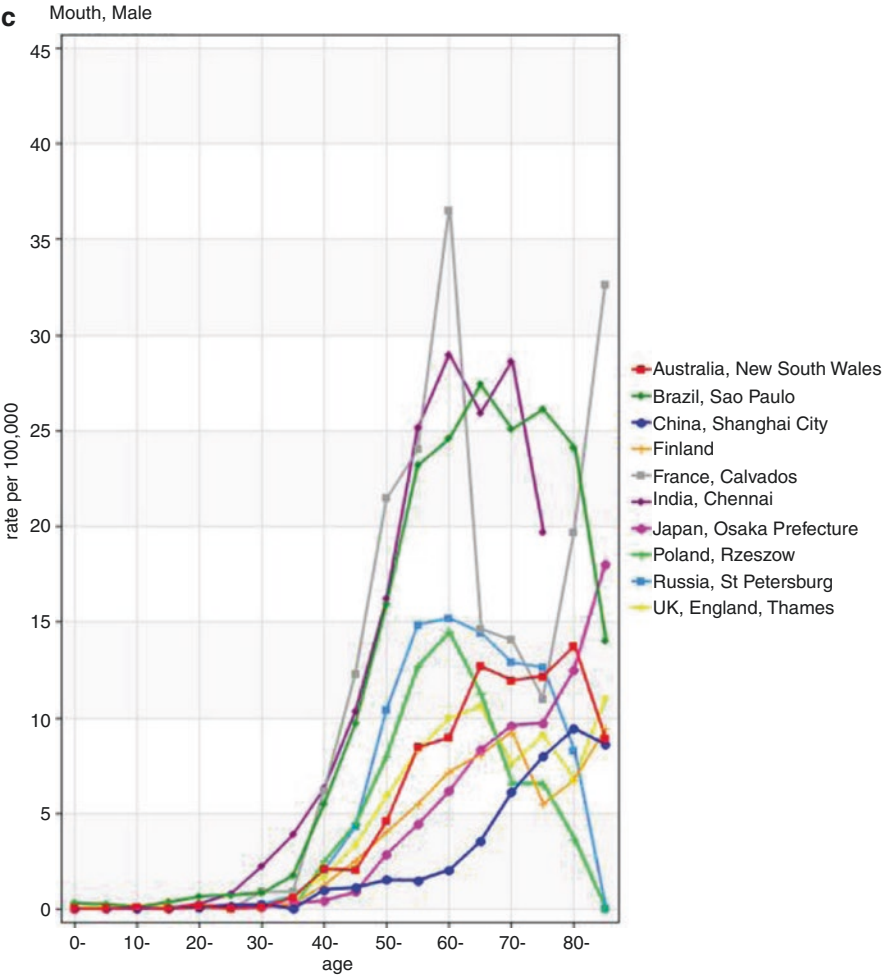

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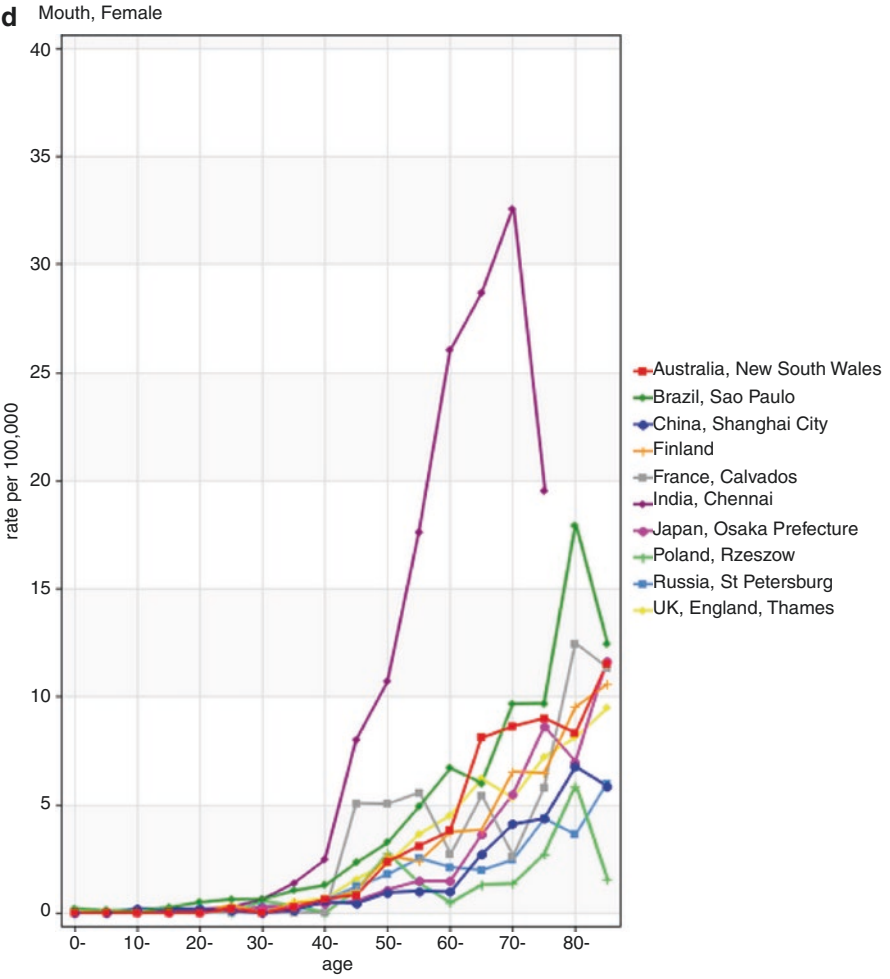


Fig. 4.7 (continued)

4.6 Mortality Trends by Birth Cohort and Forward Projections

Birth cohorts are a valuable way for interpreting time trends. Cases of particular cancers are transformed back, by convention in 5-year age groups, to the date of birth of the affected individuals. Curves derived from WHO mortality database for particularly instructive countries are given below [44]. Consistent with the evidence described above, in general these show that for most oral cancer cases, in most developed countries, rates fell in the latter part of the nineteenth and the first part of the twentieth centuries. This has been continued in, for example, the USA (Fig. 4.9a, b) and the UK (Fig. 4.10a, b). However in Hungary (Fig. 4.11a, b: and

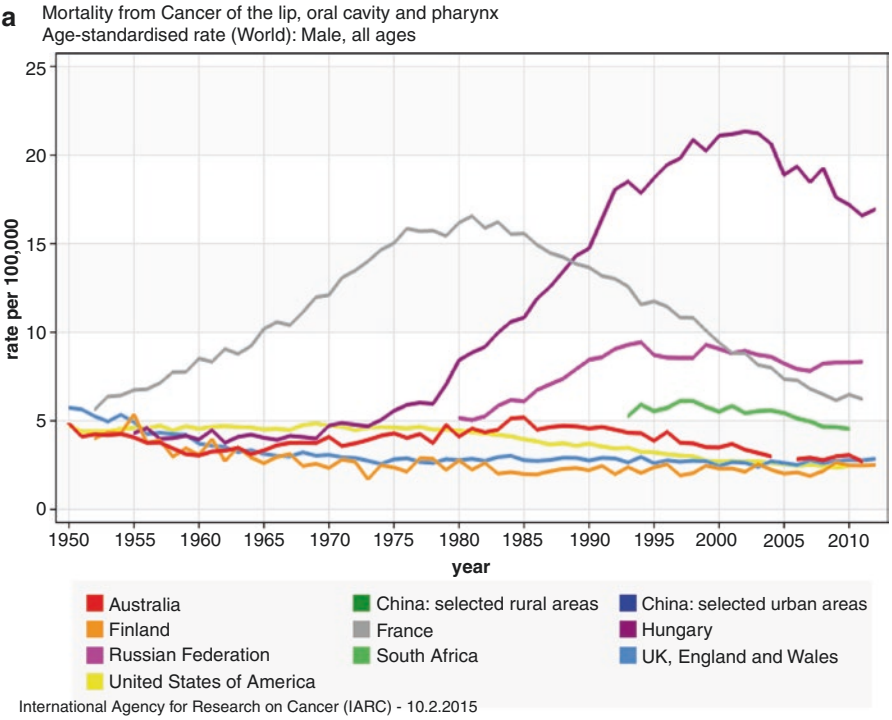


Fig. 4.8 (**a**) Trends over time in mortality from oral plus pharyngeal cancer – male. The graph covers the period 1950 to 2012. The rise in these diseases in Hungary in the period after the liberation of Eastern Europe from the mid-1970s is a disaster, though a declining trend is evident from the year 2003, this being a period with increased emphasis on health promotion. France is an example of success with rates showing a steady decline from over 15 per 100,000 pa in the 1970s and 1980s to those close to a European average by the turn of the millennium. Russia remains a concern. The overall modest downward trend in the other countries illustrated is encouraging. (**b**) Trends over time in mortality from oral plus pharyngeal cancer – female. Most of the countries shown have been relatively stable over the past 60 years. Hungary again, along with other Eastern European countries [data not shown], has been a serious concern, although at only ~a tenth of the male rate. There has been some stabilisation since the turn of the century

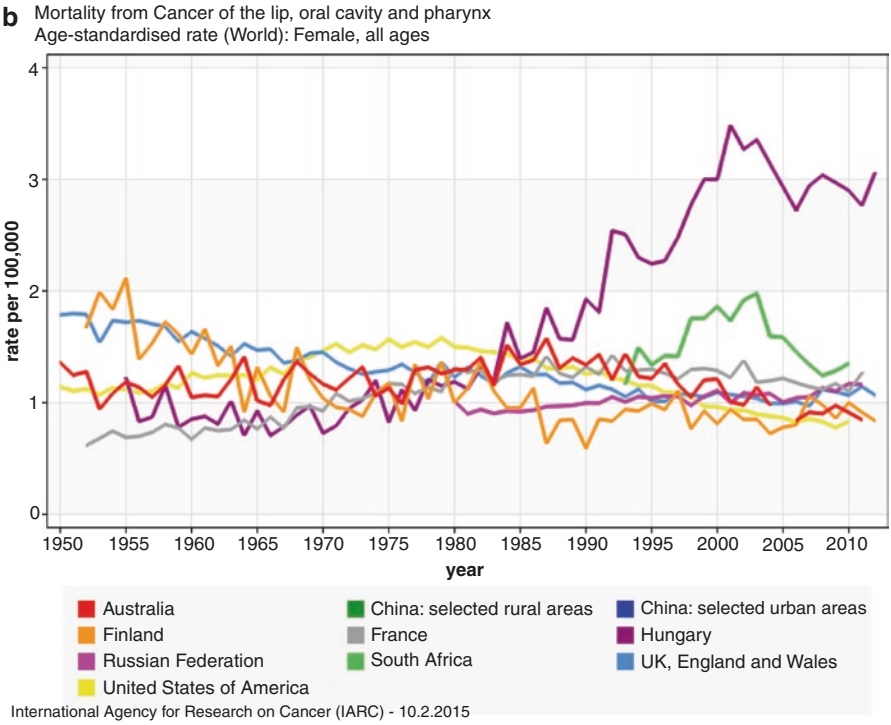


Fig. 4.8 (continued)

the same is true for most of Eastern Europe, Russia and the former Soviet Republics), those born in the first half of the twentieth century showed alarming rises in death rates. All of these birth cohorts have now passed on, or they are in the highest risk age groups: in these countries we have thus seen an epidemic of oral cancer. Indeed, ageing populations in many countries and population growth, mean that crude rates, and thus disease burden, will continue to rise, as in the data from the USA and the UK. Encouragingly, the curves now indicate that Hungary, for example, is showing control in younger people and France is a considerable success story (Fig. 4.12a, b).

The SEER programme in the USA has reported an overall fall in the mortality from oral and pharyngeal cancer, between 1975 and 2004, of 1.87 % per annum (Table 4.4). This shows a fall in all mortality rates for oral and pharyngeal cancer in the USA between 2002 and 2011. There is a considerable fall in mortality amongst both Black men and Black women (APC of −3.7 and −2.7, respectively). Furthermore, the SEER data show higher 5-year relative survival rates for Whites (64.3 %) and Blacks (43.7 %), who were diagnosed during the period 2004–2011, than rates for those who were diagnosed during the period 1974–1976 (when rates for Whites and Blacks were 55 % and 36.3 %, respectively) [46]. The 5-year survival rates in the SEER registries range from a high of 72.1 % for White women in Utah to a low of 24.8 % for Black men in metropolitan Atlanta. These striking

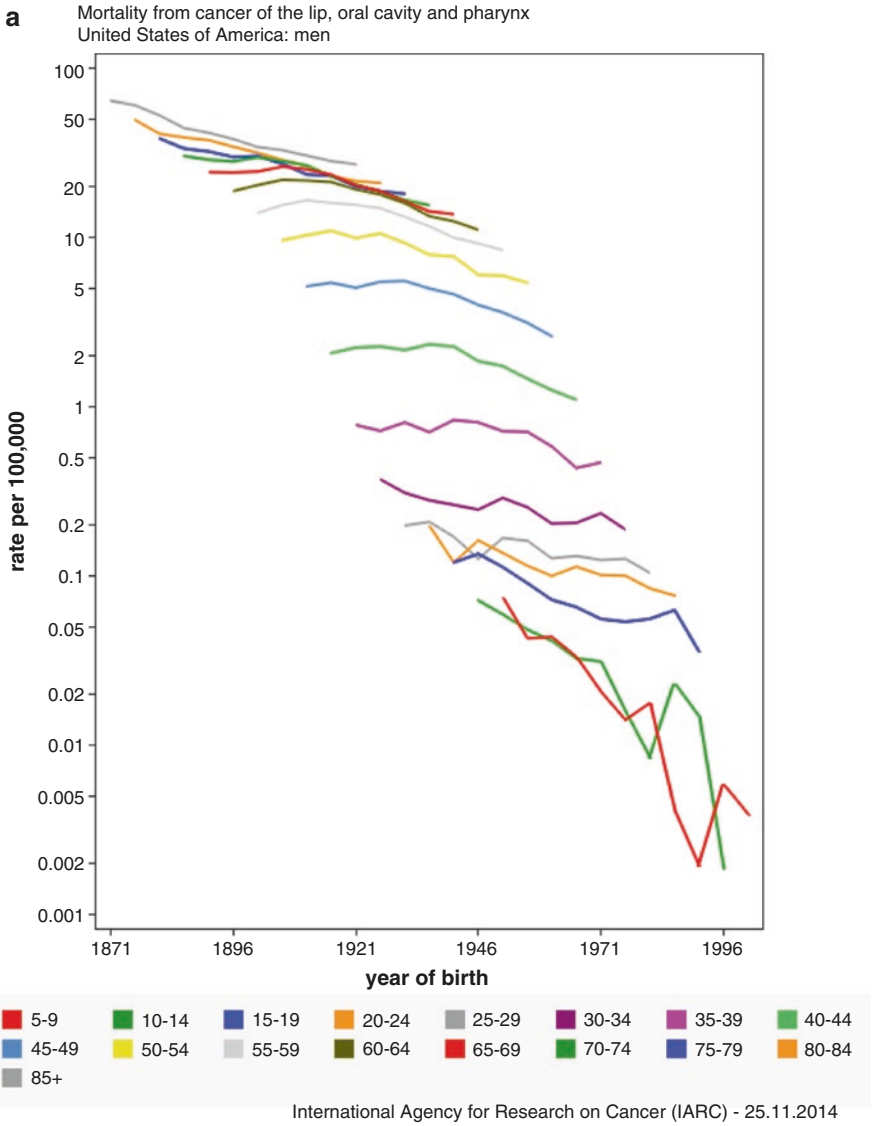


Fig. 4.9 (**a**) Mortality for oral and pharyngeal cancer in US males by birth cohort over time. Those born in the latter part of the nineteenth century and early twentieth century had very high rates, but those born from around the middle of the twentieth century, even though the oldest of these are now in high-risk age groups, have much lower rates. All birth cohorts show a steady decline. (**b**) Mortality for oral and pharyngeal cancer in US females by birth cohort over time. The much lower death rates from oral and pharyngeal cancer for women in the USA began in those born around 1920 and later. The slopes are, however, quite modest for those born in the remaining decades of the twentieth century. Data are necessarily 'noisy' for the latest born, and therefore younger, cohorts because of the smaller number of cases

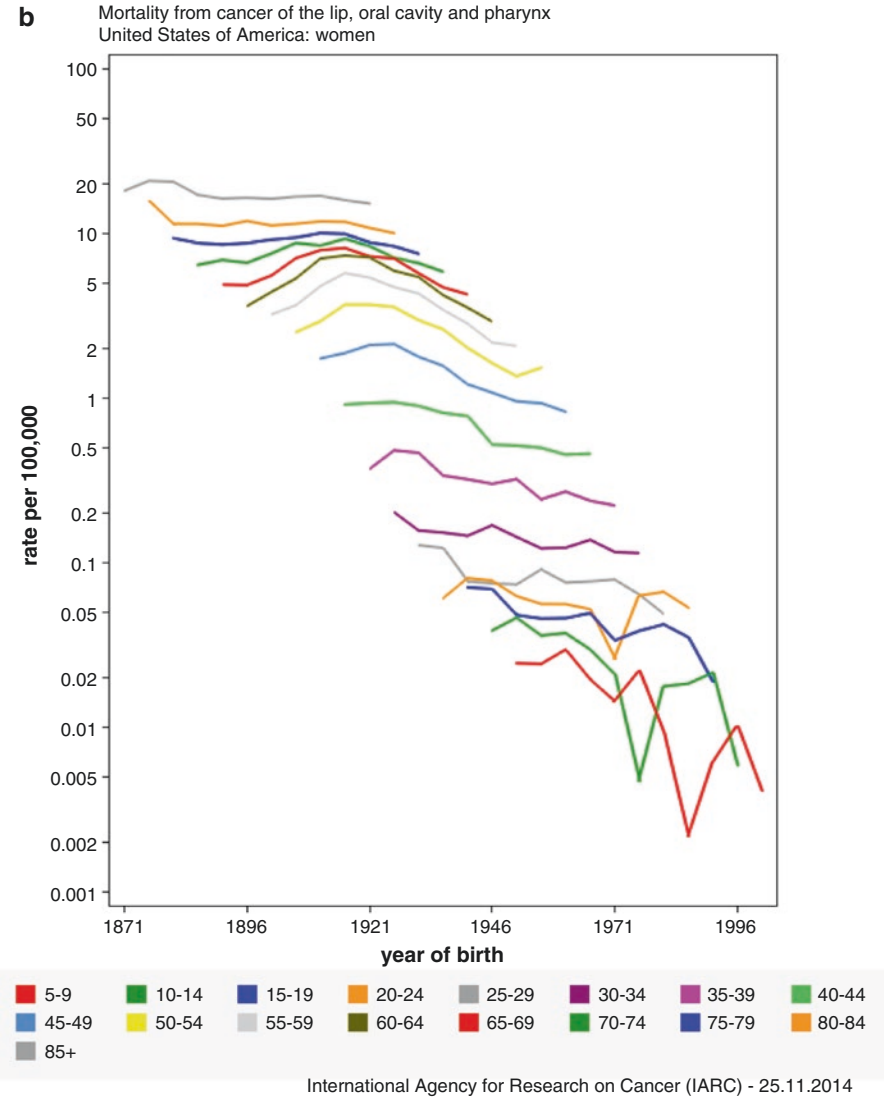


Fig. 4.9 (continued)

differences are likely to be explained by a number of factors including socio-economic condition, age, stage at diagnosis, continued presence or absence of environmental risk factors and access to hospital services. African–American patients have consistently poorer survival outcomes [47].

Forward projections for the USA (Fig. 4.13), and even more so for the UK (Fig. 4.14), show a substantially increased burden of oral cavity and pharyngeal cancers due to ageing of the population and population growth.

A study in Mumbai, India, indicated a decreasing trend in oral cancer incidence amongst Indian men, which it was suggested may be due to a decrease in the use of betel quid/pan and associated oral smokeless tobaccos over this period [48]. However, there continues to be a high prevalence of smokeless tobacco use amongst young adult men and women, especially in the form of Pan Parag/Gutka-type

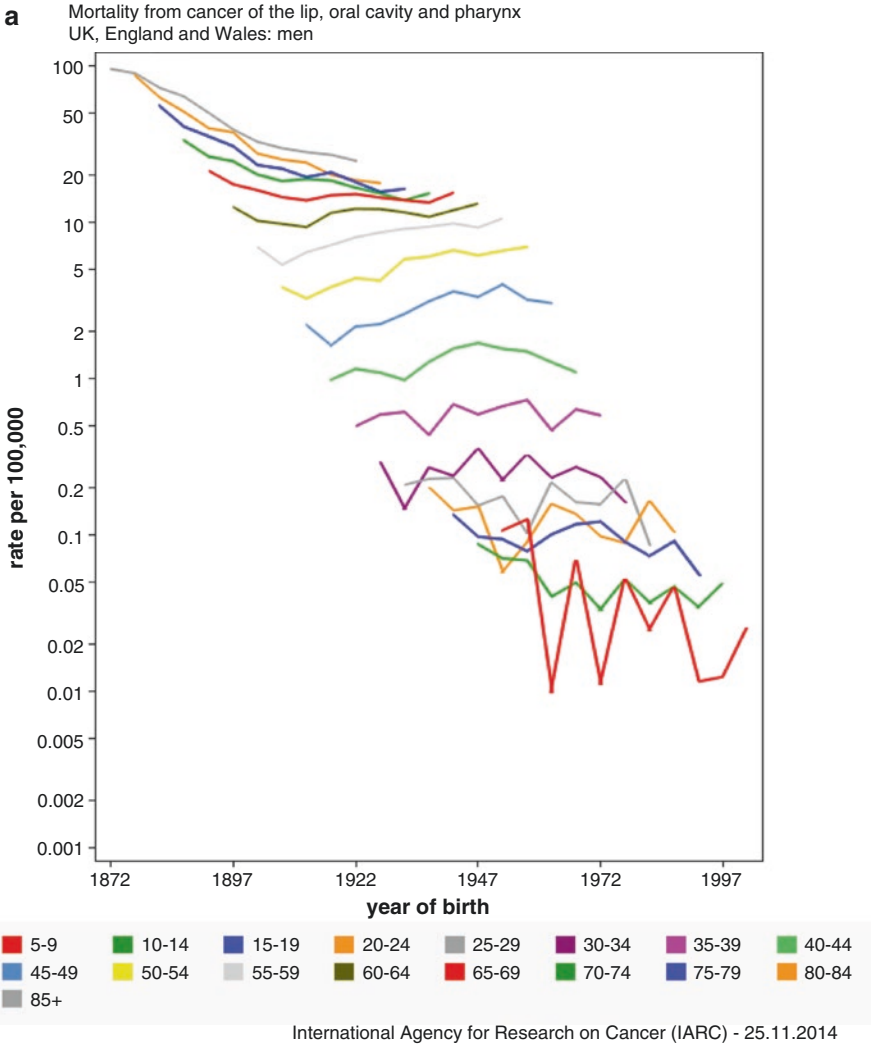


Fig. 4.10 (**a**) Mortality from oral cancer in males in the UK by birth cohort. There were declines in those born in the latter part of the nineteenth century, but unfortunately those born in much of the first half of the twentieth century show rising or stable trends. (**b**) Mortality from oral cancer in females in the UK by birth cohort. Unlike in UK males the trend is consistently downwards. However the data for younger cohorts should be interpreted with caution because of the small number of cases and consequent 'noise'

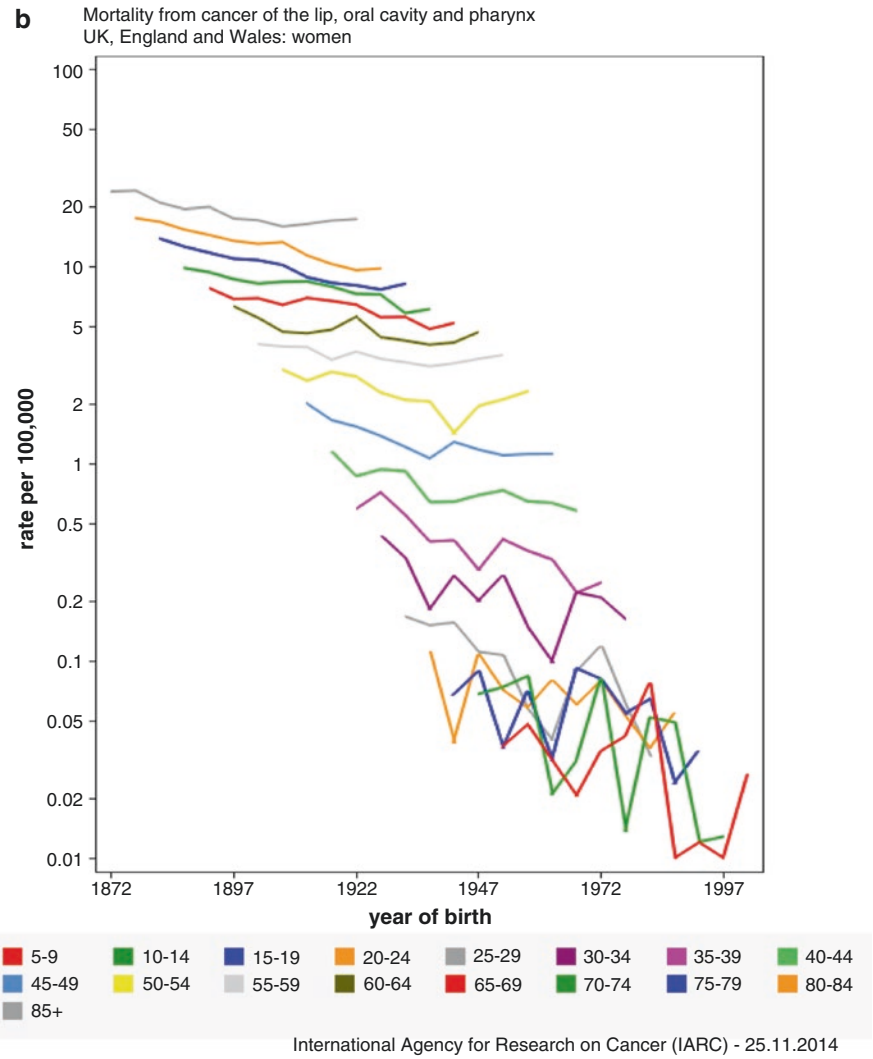


Fig. 4.10 (continued)

products, and cigarette smoking is increasing. Overall, UADT will increase, as indicated earlier [5].

Population-based survival rates around the world show little evidence of improvement over recent decades, despite vast improvements in treatment modalities. Cure rates and survival rates have improved with advances in surgical and other techniques in highly specialised, high-volume treatment institutions. Regrettably, such highly expert management is not yet uniformly available, and it may be decades before these results are reflected in population trends.

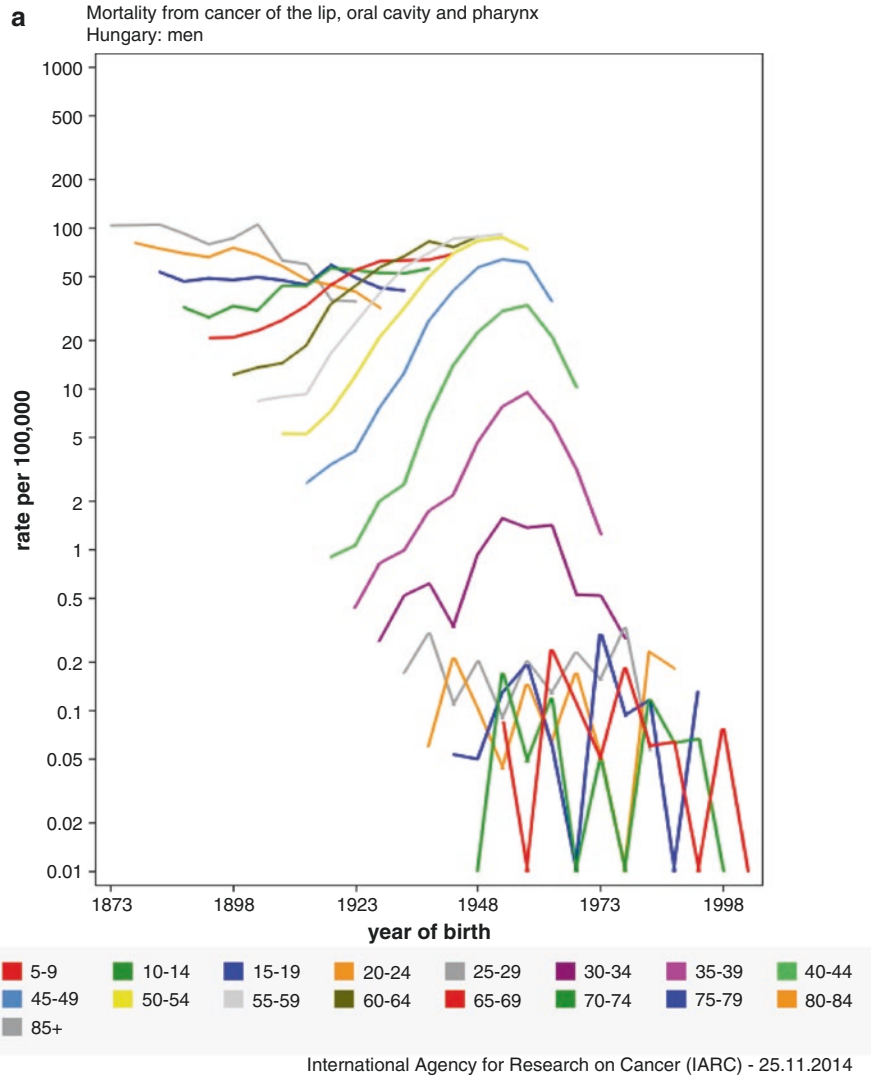
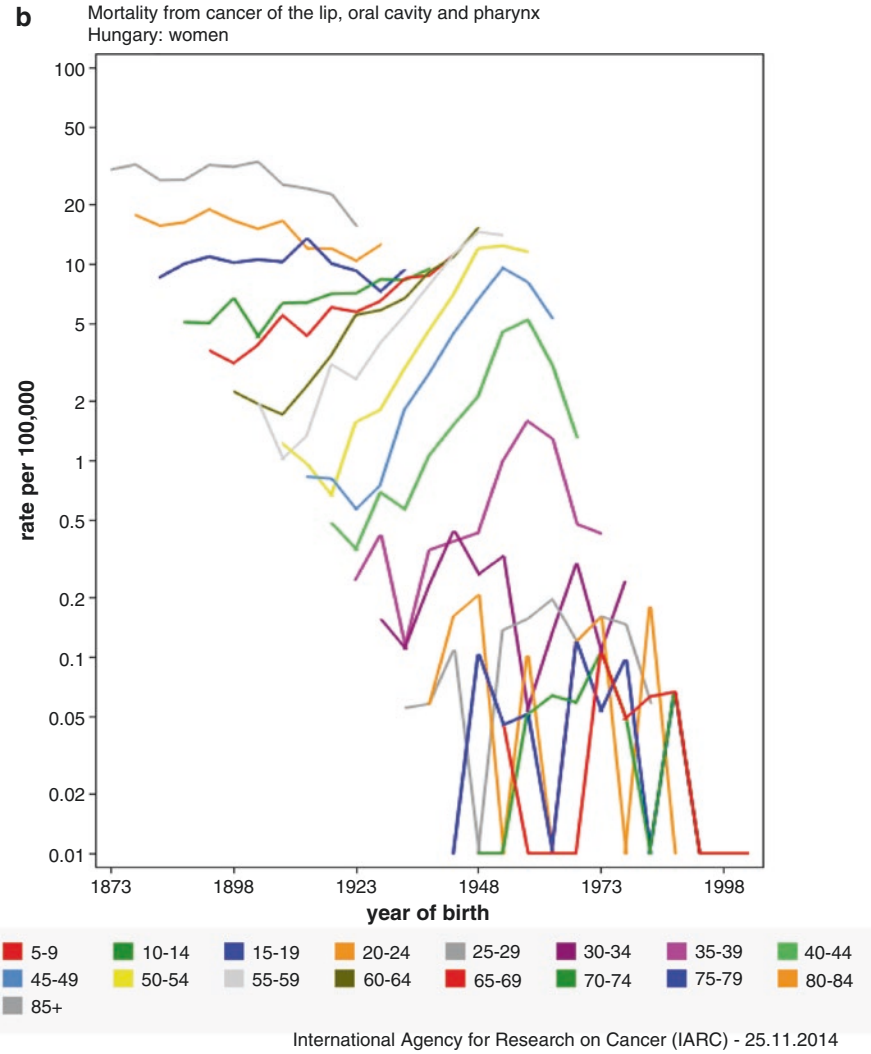


Fig. 4.11 (**a**) Mortality rates for lip, oral cavity and pharyngeal cancers for males in Hungary by birth cohort. The challenge for Hungary, apparent in other curves, is confirmed here. Males born in the first half of the twentieth century had rising rates of death from oral and pharyngeal cancer. Those born after 1950 are at less at risk. Indeed there has been a dramatic downturn. (**b**) Mortality rates for lip, oral cavity and pharyngeal cancers for females in Hungary by birth cohort. The pattern, with rates at all time periods less than half those for Hungarian men, shows a similar dramatic downturn from births around the middle of the last century onwards



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4.7 Global Scenario of Oral Potentially Malignant Disorders (OPMD)

The term oral potentially malignant disorders was recommended by an International Working Group convened by the WHO Collaborating Centre for Oral Cancer and Precancer in London in 2005 [1]. It conveys that not all disorders described under this umbrella will transform to invasive cancer – at least not within the lifespan of the affected individual. Leukoplakia, erythroplakia, oral submucous fibrosis, lichen

planus, palatal lesions in reverse smokers, actinic keratosis, discoid lupus erythematosus, dyskeratosis congenita and epidermolysis bullosa are described under the broad definition of OPMD [1, 49].

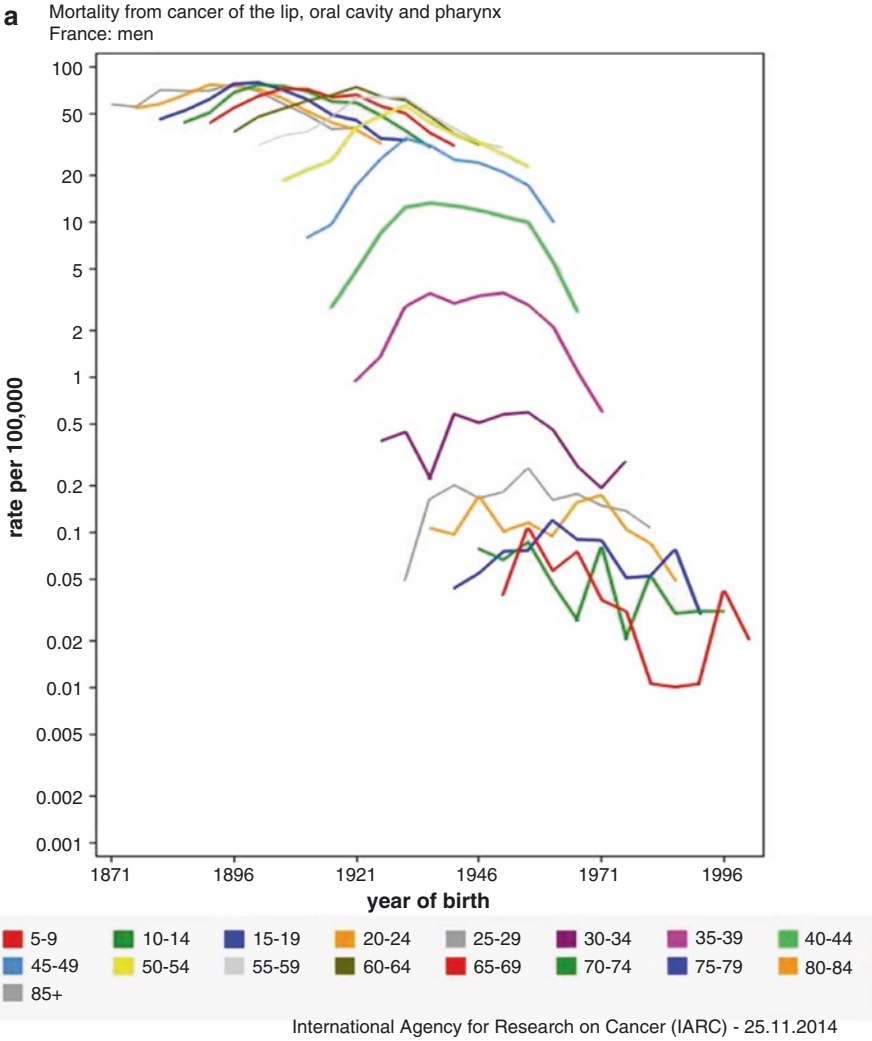
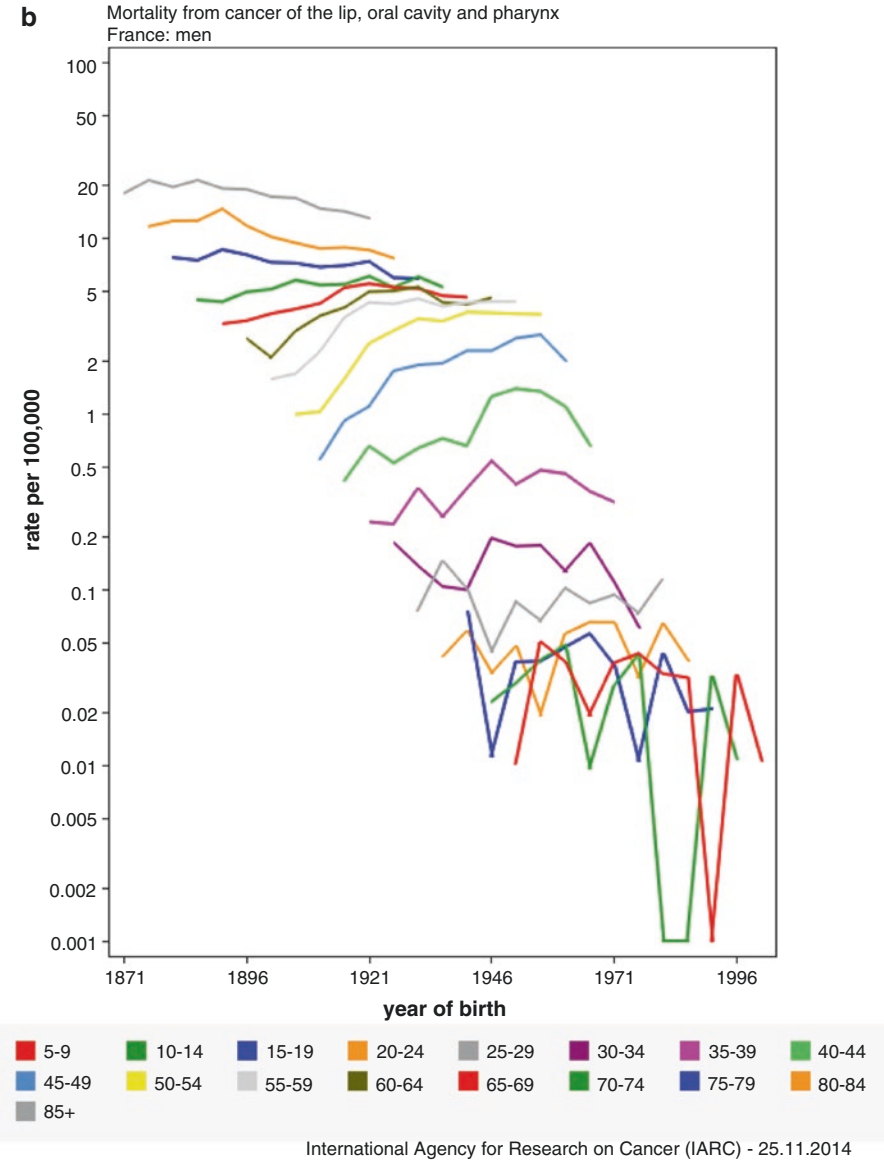


Fig. 4.12 (**a**) Mortality rates for lip, oral cavity and pharyngeal cancers for males in France, by birth cohort. For males born in the nineteenth century and the first few decades of the twentieth century, death rates from oral and pharyngeal cancer were extremely high. Those born from around 1940 and later are generating the national average downward trends seen in data presented earlier in this chapter. (**b**) Mortality rates for lip, oral cavity and pharyngeal cancers for females in France, by birth cohort. The trends are similar than those for men, from a lower base, and less dramatic



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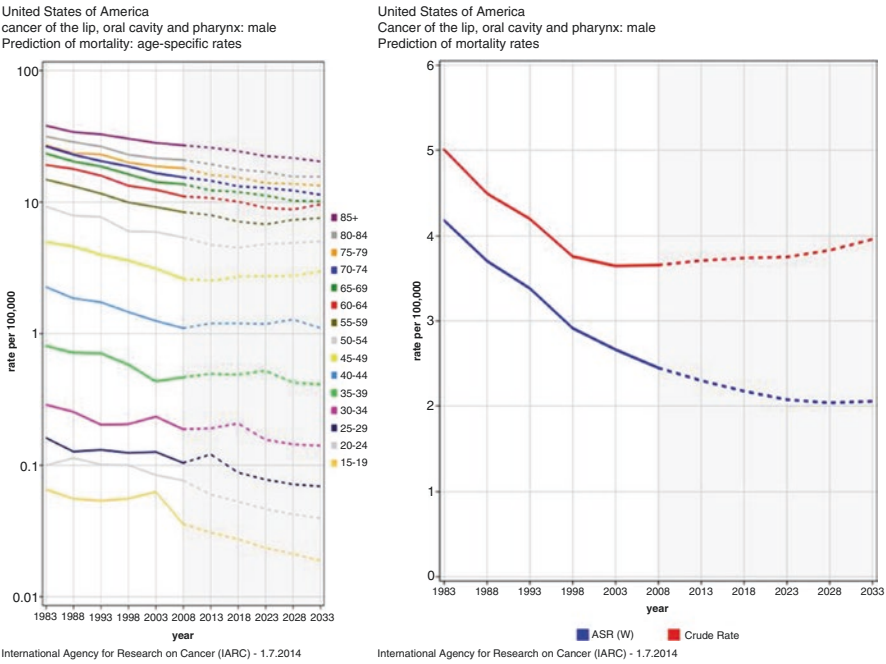

Fig. 4.13 Age-specific mortality from oral cancer in US males and projections to 2033. This presentation of the age-specific mortality rates for lip, oral cavity and pharyngeal cancers combined for US males confirms the data above. Although there are declines in all age groups, and a continuing fall in overall age-standardised rates, forward projections show rising disease burden in the decades ahead because of the ageing of the population and population growth

4.7.1 Global Prevalence of OPMD

Estimates of the global prevalence of OPMD range from 1 to 5 % [50] although much higher prevalences are reported from South-East Asia, usually with a male preponderance, e.g. in Sri Lanka (11.3 %) [51], Taiwan (12.7 %) [52] and Pacific countries like Papua New Guinea (11.7 %) [53]. Wide geographical variations across countries and regions are mainly due to differences in socio-demographic characteristics, the type and pattern of tobacco use and clinical definitions of disease (see Table 4.5). In Western countries, the overall prevalence is low and a decreasing trend over time is observed.

Petti [54] conducted a systematic review of 23 primary studies on oral leukoplakia, from international data published between 1986 and 2002. The point prevalence estimates were 1.49 % (95 % CI 1.42–1.56 %) and 2.6 % (random effect, 95 % CI 1.72–2.74 %). Leukoplakia was significantly more prevalent amongst males (prevalence ratio 3.22), but no difference was found between geographical areas and between younger and older adults. Using these data, they calculated that the crude annual oral cancer incidence rate attributable to leukoplakia would be between 6.2 and 29.1 per 100,000, implying that the global number of oral cancer cases is probably under-reported.

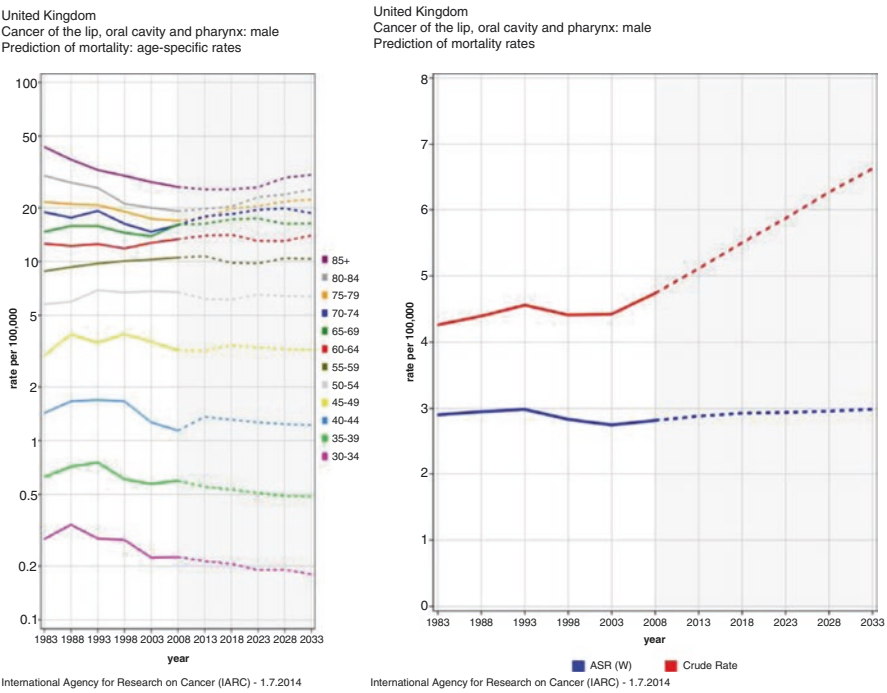

Fig. 4.14 Age-specific mortality from oral cancer UK males and forward projections. Whilst the age-standardised rates rise only slightly, the projected rises in crude rate in the years ahead, due to ageing of the population, are alarming. This will produce a growing burden of disease

4.7.2 Age and Gender Distribution of OPMD

This varies considerably, mainly dependent on lifestyle and thus on ethnicity and geographical location. In the developed world, leukoplakia is usually found between the fourth and seventh decades of life, in the developing world some 5–10 years earlier [55]. Females are less commonly affected, largely reflecting greater use of relevant habits by men.

4.7.3 Malignant Transformation of OPMD

The risk of malignant transformation varies from site to site within the mouth, from population to population and from study to study [56–58]. A classic study conducted in the 1970s with follow-up over 7 years of over 30,000 Indian villagers showed transformation rates from 10 to 24 per 100,000 per year [57]. Another classic study from the early 1980s, a hospital-based study in Californian patients with oral leukoplakia, with a mean follow-up period of 7.2 years, revealed a malignant transformation rate of 17.5 % [58]. Rates for hospital-based studies are, unsurprisingly, consistently higher than community-based studies because of sampling bias.

Table 4.5 Global prevalence of oral potentially malignant disorders

Author (Date)	Country (year)	Sampling method	M/F ratio[a]	Age group[b]	Disease entity	Definition used	Prevalence (%)
Amarasinghe et al. (2010) [51]	Sri Lanka (2008)	MSSC[c]	0.6/1.0	≥30	OPMD	WHO 1994	11.3[d]
Chung et al. (2005) [52]	Taiwan (2005)	Random	0.9/1.0	≥15	OPMD	Not given	12.7
					Leukoplakia		7.4
					Erythroplakia		1.9
					Lichen planus		2.9
					Oral submucous fibrosis		1.6
Scheifele et al. (2003) [130]	USA (2003)	MSSC	0.9/1.0	≥20	Leukoplakia	Kramer 1978	0.5–0.3
						Kramer 1980	
Ministry of Health Sri Lanka (2009) [131]	Sri Lanka (2003)	MSSC	——	35–44 and 65–74	OPMD	WHO 1994	4.1
					Leukoplakia/		2.6
					Erythroplakia		——
					OSF		0.4
Garcia-Pola Vallejo et al. (2002) [132]	Spain (2002)	Stratified, random	0.8/1.0	≥30	Leukoplakia	WHO 1978	1.6
						Axell, T et al. 1984	
Reichart (2000) [133]	Germany (2000)	Stratified, random	1.0/1.0	35–44	Leukoplakia	Axell 1976	1.6
			0.7/1.0	65–74	Leukoplakia	Zain 1995	1.0
						WHO-ICD-DA	
Nagao et al. (2000) [134]	Japan (2000)	All invited	0.4/1.0	m>40, f>20	Leukoplakia	WHO 1980	0.19
					Lichen planus		0.21
Zain et al. (1997) [135]	Malaysia (1997)	Stratified, random	0.7/1.0	≥25	Leukoplakia	WHO 1978	0.96
					Erythroplakia	Axell, T et al 1984	0.01
					OSF		0.06
					Lichen planus		0.38

Schepman et al. (1996) [136]	Netherland (1996)	Waiting room	0.9/1.0	13–93	Leukoplakia	Axell 1984 Axell 1996 Schepman 1995	0.6
Banoczy and Rigo (1991) [137]	Hungary (1991)	Random	0.7/1.0	All age groups	Leukoplakia Lichen planus	Axell 1984	1.3 0.1
Ikeda et al. (1991) [138]	Japan (1991)	Factory workers	0.5/1.0	18–63	Leukoplakia	Axell 1984	2.5
Axell and Rundquist (1987) [139]	Sweden (1987)	Stratified random	Not Found	≥15	Lichen planus	Axell 1976	1.9
Axell (1987) [140]	Sweden (1987)	All-invited residents	0.9/1.0	≥15	Leukoplakia	Axell 1976	3.6

[a]Male/female
[b]Age group (years)
[c]Multistage stratified cluster
[d]Weighted for gender and geographical location

Petti [54] has estimated a mean global prevalence of 2.6 % for leukoplakia and a mean global transformation rate of 1.36 % per year (95 % CI 0.69–2.03). Extrapolating from these figures suggests that considerably more OSCC should have been reported in recent times, a possible reason being under-reporting of cases of oral cancer in the developing world. More recently a careful study of 1,357 patients with an OPMD from the South of England revealed that 2.6 % of cases transformed to invasive cancer for a total person follow-up time of 12,273 years (mean 9.04 years): The severity of epithelial dysplasia was a significant predictor for malignant transformation [59], especially if aneuploid [60]. Similar findings come from a study of leukoplakia in Shanghai [61]. A study from a dysplasia clinic in the north of England confirms the lateral tongue as a high-risk site and that non-smokers were 7.1 times more likely to undergo malignant transformation compared to heavy smokers [62].

Controversy continues as to whether or not oral lichen planus [OLP] should be considered an OPMD. Published studies give rates of transformation from 0 to 3.5 %, over varying time periods of follow-up. A recent comprehensive systematic review evaluated 7,806 patients with OLP, amongst which a mere 85 [1.09 %] developed SCC in an average follow-up time of 51.4 months. Average age at onset of SCC was 60.8 years, with a slight female preponderance. The most common sub-site of malignant transformation was the tongue [63]. The size of any visible lesion in a subject with an OPMD is also a critical determinant of risk of malignant transformation [64].

4.8 Site-Specific Risk Factors for Oral Cancer (ICD 10, C00–C06)

It is essential to clearly define anatomical sub-sites precisely when discussing ‘oral cancer’ because aetiology, incidence rates and behaviour of neoplasms can differ substantially by sub-site. This is best achieved by adhering to the strict definitions of the WHO International Classification of Diseases 10th revision for Oncology (ICD 10-O). Thus we discuss cancers of the lip (C00); the oral or anterior two-thirds of the tongue (but not the posterior third of the tongue (C02) or the base of the tongue (C01), which is regarded as part of the oropharynx); the mucous membranes of the oral cavity proper, gum (C03); floor of mouth (C04); hard and soft palates (C05); and ‘other and unspecified parts of the mouth’ (C06). Importantly, cancers arising in the major salivary gland excluded. The tonsil, C09, and the remainder of the oropharynx, C10, should not be treated as oral cancer: together with C01, the base of tongue, these are regarded as oropharyngeal cancers. The distinction is important because many oropharyngeal cancers are related to HPV and behave as a disease very different from the alcohol- and tobacco-related cancers of the other oral sites. Over 95 % of oral cancers, thus defined, are squamous cell carcinomas histologically, and the risk factors are discussed for squamous cell carcinomas alone: we give no coverage to adenocarcinomata and lymphoid or soft connective tissue neoplasms nor to bone and odontogenic lesions.

It is members of the dental profession and wider oral health-care teams who examine mouths routinely, and who play a major role in early detection. Such clinicians ought to examine the mouth as defined above, but also including pharyngeal tonsils, in every patient be he/she symptomatic of not. They may observe the posterior wall of the oropharynx by direct vision, but cannot observe the base of the tongue or the lateral walls of the oropharynx without special equipment (mirrors or an endoscope). This chapter and these volumes as a whole concentrate on cancers of the lip and oral cavity, albeit with frequent reference to tonsillar and other oropharyngeal cancers.

As is clear from the many references throughout this chapter, risk factors for oral cancer are diverse and mainly related to risk behaviours and environmental exposures (Table 4.6) [65–67]. Oral cancer has a multifactorial aetiology, and often several factors act synergistically increasing the risk [67–69]. Based on the evidence available to date, these risk factors can be categorised into established, emerging and controversial factors. Of these, tobacco is considered the most important modifiable risk factor.

Figure 4.15 illustrates the number of men smokers across the globe. Territory size shows the proportion of men who smoke and live there. The public health burden is borne by Eastern Europe, Central and Eastern Asia and South Asia. China is the major storehouse of tobacco-related morbidity and mortality in the world, a nation where more than half the population continues to smoke. Yemen, Indonesia and Mongolia = Armenia, followed by Kenya are the top five-ranked countries for smoking prevalence, at 77, 69, 68 and 67 %, respectively. These data relate only to

Table 4.6 Summary of risk factors for oral cancer

Non-modifiable	Modifiable	Emerging	Factors with limited evidence (controversial)	Factors with no or with limited scientific evidence
Age	Tobacco smoking	Human papillomavirus infection (unsafe sexual practices)	Poor oral hygiene and dentition	Heredity
Gender	Tobacco chewing	Mate drinking	Indoor air pollution	Cannabis use
Ethnicity	Snuff use	Immunosuppression		Khat chewing
Socio-economic status	Areca nut chewing			Nicotine replacement therapy
	Excessive alcohol consumption			HIV infection
	Diet poor in fruit and vegetables			Alcohol-containing mouthwashes
	Exposure to sunlight (lip cancer only)			

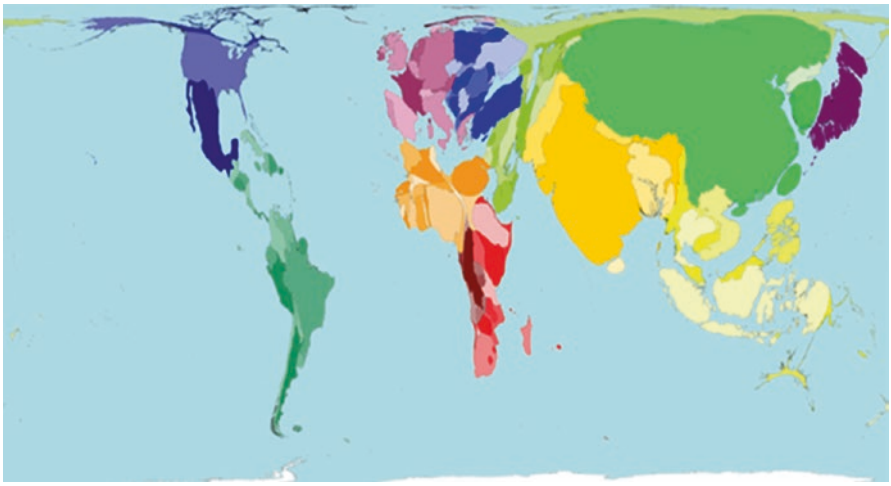

Fig. 4.15 Number of male smokers in the world. The largest population of male smokers lives in China – where men are more likely to smoke than not to smoke. Even Puerto Rico and Sweden, with the lowest percentages of men who smoke still have 17 % who are smokers. When smoking is this widespread, smokers do not just damage their own health, but also collectively damage the health of people around them. Passive smoking by children can increase the risks of asthma, cot deaths and chest infections (Available at http://www.worldmapper.org/display.php?selected=242. Accessed on 10-02-2015) ("The prevalence of smoking increased dramatically during the world wars, mainly due to the policy of providing free cigarettes to allied troops as a 'morale boosting' exercise." The Cancer Council, 2006)

smoking and do not include the many forms of oral smokeless tobacco in common use around the world. The Global Adult Tobacco Survey (GATS) is the global standard for systematic monitoring of adult tobacco use (smoking and smokeless) in the world. In India alone, the GATS (India) survey, conducted in 2009–2010 by the International Institute for Population Sciences (IIPS) Mumbai, covered about 99.9 % of the total population of India. This revealed that more than one-third (35 %) of adults in India used tobacco in some form or the other: 48 % of males and 20 % of females: 21 % adults used only smokeless tobacco, 9 % only smoke and 5 % smoke and use smokeless tobacco. Thus, in India, there are ~275 million tobacco users, 164 million users of only smokeless tobacco, 70 million only smokers and more than 42 million users of both smoking and smokeless tobacco. The distribution of tobacco use in India has been well mapped by Gupta [70].

Heavy consumption of alcohol increases the risk of oral cancer, the association showing elevated adjusted odds ratios with a clear dose–response relationship [71, 72]. Moreover, risk of oropharyngeal cancers is increased in a multiplicative or even super-multiplicative manner when alcohol and tobacco are consumed together [73]. Carcinogenic effects of the use of many forms of powdered smokeless tobacco or chewing tobacco, with or without other betel quid ingredients, have also been confirmed [74–76]. Similarly the chewing of areca nut with or without tobacco, the areca being used alone or as part of betel quid, is a prominent risk factor in South

Asian communities [19]. Whilst areca nut chewing has strong association in the pathogenesis of oral submucous fibrosis [77, 78], its carcinogenic effects in oral cancer are now well established [75]. Deficiencies in fresh fruits, non-starchy vegetables and foods rich in carotenoids contribute to an increased susceptibility to oral cancer because of the lack of protection otherwise provided by antioxidant/free radical scavenging micronutrients: approximately 10–15 % or oral cancers around the globe are associated with insufficient intake of fruits and vegetables [68, 79].

Involvement of high-risk HPV genotypes, particularly HPV 16 and 18, has been extensively studied in the recent past, and these are now established as causal for a significant proportion of cancers of the oropharynx, particularly tonsil, base of tongue and other sites of the oropharynx [80–82]. The association of HPV with oral cancer (ICD 10, C00–C06) has less frequently been identified [21]: such associations are confounded with traditional risk factors [20, 83]. Although the cause and effect relationship is yet to be elucidated, several other factors such as poor oral hygiene/high microbial load and trauma from teeth and appliances have also been implicated as risk factors in a number of studies [84–86].

Despite confounding by the common risk factors, a considerable effort has been paid in the recent past in elucidating the possible risks of oral cancer with the use of alcohol-containing mouthwashes [87–89]. A recent quantitative meta-analysis did not find any statistically significant risk of developing cancers in the oral cavity and the use of mouthwashes [90]. However, it is prudent to discourage patients from using high alcohol-containing mouthwashes on a long-term basis [91], and the controversy has resulted in many manufacturers removing high concentrations of alcohol from their products and even using this fact in advertising.

Chronic exposure to sunlight represents an important risk factor for the development of squamous cell carcinoma of the lip especially in people with fair complexions and those with outdoor occupations [92]. The lower lip is more commonly involved and intuitively receives considerably more direct sunlight than the upper lip [93]. During the last few decades, some other factors have been implicated in the development of lip, oral cavity and pharyngeal cancers which include mate drinking [94, 95], immunosuppression and organ transplantation [96, 97] and indoor air pollution [73].

Although the risk factors for oral cancer have been considered in isolation, many factors influence the pathogenesis of oral cancer in combination of two or more factors. Multiplicative effects of smoking and drinking have long been understood [73], but the true extent of such synergisms of habits, which are bound to vary by ethnicity, has been difficult to assess. A recent meta-analysis of observational studies from South-East Asia reported pooled ORs for smoking, drinking, chewing and smoking-drinking-chewing, respectively, at 3.6, 2.2, 7.9 and 40.1, all of which are statistically significant. Amongst all three habits, the individual effects accounted for 6.7 % (smoking), 3.1 % (drinking) and 17.7 % (chewing) of the risk, the interaction effect accounting for the remaining 72.6 %. Some 44,200 oral cancer cases in South-East Asia annually occur amongst smoking-drinking-chewing exposed subjects, and 40,400 of these are exclusively associated with the interaction effect [98].

4.8.1 Lip Cancer (ICD 10, C00)

Squamous cell carcinoma of the lip is a frequent neoplasm of the head and neck. Depending on the population studied, lip cancer represents between 25 and 65 % of all malignant neoplasms of the head and neck, particularly in the Western world [99, 100]. Lip cancer is commonly seen amongst men with a male-to-female ratio of 5:1, the lower lip being affected more commonly than the upper [101]. Although risk factors for lip cancers are notably different from the rest of the mouth, it is often grouped together with the oral cavity and pharynx in epidemiological studies.

It is well known that chronic exposure to ultraviolet radiation from sunlight is the major cause of lip cancer. The lower lip is more commonly involved because it receives considerably more direct sunlight than the upper lip [93].

Evidence comes from several countries, including those at latitudes with clean air through which ultraviolet light penetrates easily, such as Finland [102] or Sweden [103]. Similarly, in countries closer to the equator with regular long hours of sunshine such as rural Greece, lip cancer can account for 60 % of oral cancers [104] and is common amongst fishermen in India [105]. All outdoor occupations which expose individuals to prolonged periods of solar radiation place them at higher risk. A cumulative effect of sun exposure amongst individuals in fisheries- and agriculture-related occupations has been demonstrated [106].

Lip cancer is typically a disease of old age and amongst men [93]. This gender bias is related to social behaviour, as men work more outdoors. Further, frequent use of lipstick may be protective [107]. Although exposure to sunlight is the major risk factor, it is often confounded with smoking and alcohol. A study in Finland demonstrated that the risk for lip cancer is confounded by smoking and social class [102]. A case–control study in Southern Spain demonstrated similar confounding with tobacco and alcohol consumption [106].

Lip cancer is more common amongst people with fair complexion and rare amongst people with dark skin. In a population-based case–control study of lip cancers in the multicultural population of California, Pogoda and Preston-Martin showed a statistically significant association between lip cancer and skin complexion [107].

The level of exposure to solar ultraviolet radiation varies with latitude, altitude, time of day, time of year, cloud cover and reflection from nearby surfaces [108].

Interpretation of the epidemiological data is difficult due to many confounding factors. Maruccia et al., in a retrospective Italian sample, identified smoking as a strong risk factor for lip cancer [93]. An epidemiological study in Southern Spain found that the habit of leaving the cigarette on the lip whilst smoking increases the risk, which is independent of the cumulative effect of the amount of tobacco smoked [106].

Immunosuppression has been shown to increase the risk of lip cancer. The risk is 44 times higher amongst solid organ transplant patients, kidney transplant recipients having the highest risk [97]. The risk is directly related to the immunosuppressive regime and is reversible when the therapy is stopped [109]. The incidence of lip cancer amongst renal transplant recipients is directly related to the type of

immunosuppressive therapy, dosage and duration [109]. The risk of cancer development on the lower lip vermillion in renal transplant individuals is independently related to increasing age, smoking, exposure to solar UV radiation and the duration of immunosuppression [110]: patients with solid organ transplants and on immunosuppressive therapy should avoid exposure to sunlight, heavy alcohol consumption and smoking [109]. HIV/AIDS patients carry a higher risk of HPV-related cancers of the cervix, anus and skin and in the head and neck; a recent meta-analysis has shown an increased risk of lip cancer amongst these patients [96], but this is substantially lower than for patients undergoing immunosuppressive therapy.

There is increasing attention on possible side effects of commonly used pharmaceutical agents in their putative role in the development of lip cancer [111]. A recent study conducted amongst non-Hispanic males revealed an increased risk of lip cancer amongst patients on photosensitising antihypertensive drugs, including thiazide diuretics, triamterene and some angiotensin-converting enzyme inhibitors [111].

4.8.2 Cancer of the Oral Tongue (C001–C002)

The tongue is the most common intra-oral site for squamous cell carcinoma amongst European and North American populations, accounting for 40–50 % of all oral cancers [66, 112]. Of the tongue, the lateral border and base of the tongue are the most cancer prone sites [55]. Tobacco smoking and excessive alcohol consumption are the most important risk factors for cancer of the anterior two-thirds of the tongue [113–116]. A case–control study conducted in Beijing, China, revealed that the risk of tongue cancers was significantly elevated amongst ex-smokers (OR = 2.14) and amongst current smokers (OR = 2.73). Smoking affects in a dose-dependent manner. Persons who smoked more than 20 pack-years of cigarettes carry the highest risk (OR = 5.06). Quitting tobacco was associated with reduction of the risk, but the number of subjects in the study was too small to define the time period necessary for substantial reduction [116]. Surprisingly, this study did not find significant associations with alcohol consumption, though drinking spirits at least 5 days a week was marginally significant.

Although the exact reason is yet to be elucidated, tongue cancers are becoming more common amongst the young [60]. A retrospective study on oral cancer patients in Sri Lanka revealed that 5 % of the cancers of their sample were amongst the young [117]. Of this, the tongue was the most common site (41 %) [118]. A study in India, seeking to ascertain risk factors for intra-oral sub-sites, found that tongue cancer was the most prevalent in their cohort, but the authors were unable to identify patient-related characteristics involved with greater risk in developing these lesions [84].

Another study in Italy found that tongue cancers were more common amongst non-smokers compared to all other sites. This highlights the fact that there are other unknown risk factors involved in the pathogenesis of tongue cancers [119].

It is generally regarded that viruses are implicated in 10–20 % of all human cancers, including DNA oncogenic viruses such as some HPVs and HHVs. A number

of recent studies have emphasised the aetiologic role of HPV in the pathogenesis of oral cavity cancers, particularly the tonsil and the base of the tongue [80–82]. Malignant transformation in the presence of persistent HPV infection is distinct when the common behavioural risk factors are low or absent, although on most occasions the influence of HPV is confounded by the common risk factors.

Infection with high-risk HPV genotypes, particularly HPV 16 and 18, has been extensively studied and causally related to the base of tongue cancer [80–82]. A study conducted in North Carolina, USA, has demonstrated that the odds of having HPV infection in tumours of the base of the tongue amongst non-smoking non-alcohol-consuming patients was significantly higher than in control patients [81].

Dahlgren and colleagues reported that HPV was detected in 10 out of 25 (40 %) base of tongue carcinomas and rarely (2.3 %) in carcinomas of the anterior two-thirds of the tongue [120, 121]. Although cancers of the mobile tongue are increasing, especially amongst the young, the pathogenesis cannot be explained due to the frequent absence of traditional risk factors. Moreover, the cumulative exposure to smoking and alcohol is comparatively low amongst young patients. It has been suggested that increased HPV infection rates in the oropharynx are related to changing sexual behaviours, with more frequent oral–genital contact [122]. Virus can also be transferred on fingers or fomites such as sex toys. HPV-related oral and oropharyngeal cancers are more common in Western societies and amongst the young.

4.8.3 Gum Cancer (ICD 10, C03)

The most common risk factors for the development of cancers on the gingivae are yet again tobacco smoking and chewing and heavy alcohol consumption [119, 123]. Unfortunately much of the data do not make a distinction between intra-oral sites. A recent case–control study in France identified risk factors for cancers of oral subsites: this revealed that smoking and heavy alcohol consumption (defined as greater than or equal to two glasses per day) and the combination of both have significant, super-multiplicative effects for gum cancers. Current smokers who smoke more than 20 cigarettes a day had a higher risk than non-smokers (OR = 2.9), whereas those who smoked for more than 30 years had the highest risk (OR = 3.8), compared to non-smokers. In regard to alcohol consumption, the risk was lower than that of smoking and not significant even for individuals who drink more than two glasses per day [123].

A case series analysis in the USA found that the gingiva was the third most common site for squamous cell carcinoma in the mouth [124] and suggested a different mix of risk factors for gingival carcinoma in comparison to other intra-oral sites. A case–control study in Kerala, India, on cancers of the gingiva revealed tobacco chewing and alcohol consumption as significant risk factors. Amongst males a significant positive association was found for chewing of betel quid with tobacco ($p < 0.001$), bidi smoking ($p < 0.001$), alcohol consumption ($p < 0.001$) and snuff use ($p < 0.001$). Amongst females there was a similar relationship with chewing of betel quid containing tobacco. All these habits influenced in a dose-dependent manner.

Daily frequency of paan–tobacco chewing was the strongest predictor in males, a relative risk of 15.1 being associated with chewing ten or more quids per day [125].

4.8.4 Cancers of the Floor of the Mouth (ICD 10, C04)

Smoking and excessive alcohol consumption are by far the most important risk factors for cancers of the floor of the mouth. A recent case–control study in France revealed that this is the most susceptible site in smokers and heavy alcohol consumers [67, 123]. The combined effect of smoking and alcohol is greater in the floor of the mouth than in other intra-oral sub-sites ($p = 0.005$) [119].

Current smokers carry the highest risk to develop floor of the mouth cancer and the risk increases in a dose-dependent manner. Compared to non-smokers, smokers for 1–30 years carry a higher risk with an odds ratio of 29.2 amongst those who smoke 20 or more cigarettes a day: those who smoke for over 30 years and over 30 cigarettes a day have the most risk, with an odds ratio of 85.3. The odds ratios for the other sites are comparatively low, highlighting the pronounced effects of smoking on the floor of the mouth [123]. Similarly, the floor of the mouth is the most vulnerable site for cancers amongst those who drink alcohol (OR = 3.4) more than two glasses a day [123].

Dhar et al. (2000), in a study conducted in India, reported that alcohol and smoking are the high-risk factors for the floor of the mouth in comparison to all other sub-sites grouped together, with ORs of 1.81 and 1.76, respectively [115]. The tissue characteristics in the floor of the mouth may contribute to this effect: this region has a thin vascular mucosa which is highly permeable, and alcohol and tobacco carcinogens dissolved in saliva pool here [118].

4.8.5 Carcinoma of the Palate (ICD 10, C05)

Sub-sites of the palate include the hard palate (C05.0), soft palate (C05.1) excluding nasopharyngeal surface of the soft palate, uvula (C05.2), overlapping lesions of the palate (C05.2) and palate unspecified (C05.9). Tobacco smoking and alcohol are yet again the most important risk factors and their effects are super-multiplicative [119]. Some studies show that the effects of smoking and alcohol consumption do not differ between anatomical sub-sites of the oral cavity [123]. On the other hand, in a study in France, smoking was found to be the most important risk factor for SCC of the soft palate. This study revealed that smoking affects the soft palate in a dose-dependent manner with increasing odds when the number of cigarettes per day and the duration of smoking increase [123]. A similar pattern has been observed for alcohol consumption, but the risk was relatively low (OR = 1.7). This study computed population attributable risk for palatal carcinoma as 2.9 % for alcohol and 83.8 % for tobacco smoking. Such precise figures will, of course, be population specific, depending on the nature and prevalence of relevant habits and the toxicity of the most common forms of tobacco.

HPV can also play a major role in developing carcinoma at this site. A recent systematic review and meta-analysis has shown that the palate is less commonly affected by HPV than the palatine tonsils and the base of the tongue. However, this analysis was based on a small sample and sub-site misclassification may have influenced the conclusions: many cases had been classified as 'palate non-specific' and as 'palate' and the involvement of HPV in palate non-specified sites was high [83]. According to this study, the prevalences of HPV (HPV 16 and other types) were 6.2 % for the hard palate (C05.0), 11.7 % for the soft palate (C05.1) and 42.6 % for the palate unspecified (42.6 %) [83].

4.8.6 Carcinoma of the Other and Unspecified Parts of the Mouth (ICD 10, C06)

Sub-sites of C06 include cheek mucosa (C06.0), vestibule of the mouth (C06.1), the retro-molar area (C0.2), overlapping lesion of other and unspecified parts of mouth (C06.8) and mouth unspecified (C06.9). Carcinoma of the buccal mucosa is mainly discussed here. All major risk factors mentioned above are involved in the initiation of carcinomas of the buccal mucosa. The few studies that have conducted analysis of sub-sites have demonstrated that smoking and alcohol are again the major risk factors. Pentenero et al. (2011) [119] reported that smoking carries an OR of 4.5 but the effect was not statistically significant. There is a geographical variation on the risk factors for SCC at this site. In the USA tobacco smoking and excessive alcohol consumption are the major risk factors [28], whereas in South Asia the major risk factors are chewing betel quid, especially containing tobacco, and areca nut [67, 115].

A betel quid usually contains betel leaf, areca nut and slaked lime and may or may not contain tobacco. In addition other substances such as cardamom, saffron, cloves, aniseed, turmeric, mustard or sweeteners are added according to local preference [75]. Betel chewers usually keep the quid in the buccal sulcus one side or both and some sleep with the quid in the mouth.

Chewing tobacco is prepared from sun dried and partly fermented, coarsely cut leaves of Nicotiana rustica and/or Nicotiana tabacum without further processing. Tobacco chewing results in a local exposure of the oral mucosa to at least 28 carcinogens, including tobacco-specific nitrosamines (TSNA) and polycyclic aromatic hydrocarbons (PAH) [68].

Snuff dipping is also a common practice in some parts of the world. The product known as Toombak in the Sudan and Shammah in Yemen and Southern Saudi Arabia is particularly toxic. High levels of carcinogenic TSNAs (e.g. NNN, *N*-nitrosonornicotine and NNK, nicotine-derived nitrosamine ketone) are reported in the saliva of oral snuff users [126] and tobacco chewers [127]. NNK is a potent carcinogen, and in vitro studies on human buccal epithelial cells have shown that they metabolise NNK and form macromolecular DNA adducts, the concentration of which is correlated with carcinogenesis in animal models [128].

Areca nut itself is now recognised by the International Agency for Research on Cancer as a Class 1 human carcinogen [129]. Chewing betel quid also releases large

amounts of a reactive oxygen species (ROS). Both TSNA and ROS are major genotoxic agents involved in chewing tobacco-associated oral cancer [75]. Clear dose–response relationships between quid use and the risk of oral cancer and of oral potentially malignant disorders have been demonstrated in many epidemiological studies.

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Histopathology of Oral Cavity Cancer and Potentially Malignant Disorders

5

Mihai Merzianu

5.1 Introduction

Oral carcinoma is a devastating disease with only minimal improvement in survival in the last decades despite progress made both in the understanding of molecular alterations involved and in the array of therapeutic options available. The most common malignant neoplasm is squamous cell carcinoma comprising over 90 % of oral cancers. Squamous cell carcinoma is an extremely heterogeneous neoplasm and probably in no other oncologic field collaboration between various specialists (surgeon, dentist, medical oncologist, radiation oncologist, pathologist) is as critical for the correct diagnosis, staging, and accurate characterization of the tumor essential for the appropriate management with the best chance for cure.

5.1.1 Pathology Practice, the Pathologist, and Surgical Pathology Report

The role of the pathologist in general and in the care of patient with oral cancer in particular remains largely unknown to the general public and unfortunately to a certain number of clinical practitioners. It is well recognized that the pathologist is a critical member of the oncologic team [1, 2], but the situation in a small practice may be different. Solo practitioners may face different challenges than the hospital-based pathologist simply in gaining access to, and communicating with, the clinician at a different hospital, clinic, or office.

The pathologist functions as a consultant for the surgeon and other members of the clinical team (radiation oncologist, medical oncologist) and is expected to render a comprehensive yet timely report encompassing all the elements allowing the

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treating physician to decide on the best therapeutic approach. Tumor type, completeness of excision, pathologic staging, and presence or absence of various histologic risk factors – all may impact prognosis, therapy, or both.

Even at the time of biopsy evaluation, the amount of information that pathologist can render from a 3 mm fragment of tissue is staggering [3], and a single finding (e.g., perineural invasion) may significantly alter the therapeutic decision.

The surgeon and pathologists' worlds are quite different, with brief intersections during intraoperative consultation, when discussing a surgical specimen or the pathology report, and, where available, tumor boards or multidisciplinary conferences. Details of surgical techniques and challenges remain mostly unknown to the general pathologist or even to the head and neck or oral specialists. Vice versa, most surgeons are not privy to the pathology processing or evaluation methods, their indications, and limitations. Many recently trained surgeons seem to think that pathology is some magical machine, where tissue samples are somehow converted onto diagnostic reports ("biopsy was sent to pathology and came back as benign" is a common rendition in the United States). Encounters where the pathologists "need more tissue" for a diagnosis, stemming probably more from individual uncertainty than sample sufficiency, can also be frustrating to the operator.

Historically, surgical pathology was developed by and for the surgeons [4], and until four to five decades ago, the surgeons in training spent up to 6 months rotating through and doing benchwork in pathology, gaining thus first-hand knowledge and hands-on experience in the pathology practice, processes involved, and specialty's strengths and limitations. After the advent of subspecialization, with its multiple incontrovertible benefits for the trainee, attending physician, and patient, the rift between specialties has become deeper as signaled by others two decades ago [5] when the rotation of the surgeons in pathology rotation was lamented of being "dashed through in a 1-month scramble or left entirely optional." Same authors would be dismayed to witness the state of affairs today when surgical training programs have no formal requirement for a minimum pathology exposure [6] and, if the pathology rotation option is presented in individual programs, most trainees do not take advantage of it. It is safe to say that after inventing and developing the discipline of surgical pathology, the surgeons completely withdrew from its practice. To compound the issue, in the United States medical students are offered an *elective* rotation through pathology, which is studied in a modular system embedded with all other clinical and preclinical disciplines for each organ and system site.

What is important for a surgeon or dentist may seem trivial to the pathologist as this observer has often witnessed during multidisciplinary conferences, but in a consultation and referral practice, one also sees pathologists agonizing over a detail that may be clinically irrelevant. Sometimes pathologists and clinicians do not agree to what a critical value is and a recent consensus paper clarified the pathologists' position on the matter [7]. Terminology used in the pathology reports and literature is often unclear to the clinician, and an attempt will be made below to clarify some concepts, illustrate technical difficulties, or translate pathologic jargon (e.g., "tangential orientation"). Regular meetings, such as tumor boards, and/or continuing patient-related communication bring the two worlds together, allowing essential, mutually beneficial education and, most importantly, benefitting the patient care.

Shortly after a specimen is received in the pathology department, prompt communication with the surgeon for questions on orientation, anatomic landmarks, or margins may be needed. Specimens are distorted *ex vivo* and it is not uncommon for experienced attending surgeons to have difficulty answering anatomic questions in the specimen they just removed. Bisecting a tumor or sectioning the specimen looking for the tumor in the operating room should be avoided since it may compromise surgical margin assessment [1].

The responsibilities of the pathologist in patient management start when the patient is in the operating room with an intraoperative consultation which may change the entire planned procedure and end with the pathology report which should be concise yet comprehensive, clearly listing all the features of the tumor that will allow optimal management [2].

However, the pathologist cannot reach the ultimate goal without the surgeon's familiarity with the entire process. Regardless of the training and experience of the pathologist reading a slide, a completely necrotic tissue will be equally nondiagnostic for the novice and the expert.

Correct and complete gross evaluation, assessment, and adequate sampling, entirely the responsibility of that pathologist, are all indispensable for a correct diagnosis. General gross evaluation principles are beyond the scope of this text and well illustrated elsewhere [8, 9], but a minimalist approach to sampling at times championed by some pathologists or due to other local factors may be detrimental in detecting subtle or focal disease, a microscopic focus of cancer, deepest area of involvement, perineural spread, etc. Examining several levels of oral mucosal biopsies is currently not mandatory in general clinical practice but is routinely done in many laboratories, including ours, to prevent sampling error and better control for lesion heterogeneity and recommended for mucosal high-risk lesions or sites [10]. Appropriate use of ancillary studies usually falls under pathologist purview, although at times the surgeon or other clinician will request it. There is currently no consensus on how should one proceed if the requested test is deemed unnecessary.

Systematic evaluation and reporting of all required information for cancer patient management includes rendering a timely, definitive, and precise diagnosis, an accurate pathologic staging, assessing resection completeness, and listing the histologic parameters of prognostic import.

Due to clinical practice diversity, several national or regional organizations have published consensus guidelines to ensure that a minimum of information for oral cancer is generated for each case regardless of local practice setting or individual pathologist's preference [11, 12].

A pathologic diagnosis is an integrative process of histologic findings in the clinical context. It is important to highlight the essential roles and responsibilities of the clinician (surgeon or dentist) in the successful diagnostic process: providing pertinent clinical history, correct anatomic localization and clinical description of the lesion, adequate sampling and handling the specimen until its receipt by the pathologist, and communication openness. Recording the clinical impression or differential diagnostic considerations in the requisition form seems trivial but is often absent in routine clinical practice and may hamper or mislead the pathologic evaluation with significant consequences. Informing the pathologist on potential

confounding factors (prior local radiotherapy, previous carcinoma, immunosuppression, etc.) may trigger a different workup or alter one's interpretation of the same histologic findings often to dramatic degree: benign versus malignant cytologic atypia is a common quandary both for the clinician and pathologist. Optimal sampling from the most representative area, quantity and quality of the biopsies (deep enough sample, away from the necrotic center, ideally with interface between normal and lesional tissue), adequate labeling and identification are essential. Clinician's availability to openly communicate with, or respond to, the pathologist call or inquiry is also critical. Clinical description of the pathologic process sampled should be clear and descriptive (e.g., plaque, ulcerated mass, polypoid tumor), the site precisely designated (e.g., left anterior third lateral tongue), whereas vague descriptions of site and appearance (e.g., tongue lesion) provide little useful information for the pathologist diagnostic algorithm and the patient records. Since the pathology report is a medicolegal document, documentation of the center of lesion and biopsy site – not necessarily identical – is critical. As it will be discussed, base of tongue cancer has today different etiology and prognosis than oral tongue cancer and should be clearly designated by the surgeon. Depending on the lesion, one biopsy usually suffices; however, if broader lesions are present, multiple biopsies may be needed in identifying the most aggressive tumor focus or highest grade of dysplasia. Integrity of excisional and resection specimens is important, and bisecting or sectioning the tumor in the operating room may compromise accurate assessment of the resection margins or tumor's relationship with adjacent structures.

The pathology report should provide basic information about the nature of the procedure, tumor type, grade, size, site, gross appearance, focality, extent, and relationship with nearby structures, nodal status, excision completeness, as well as other important histologic prognosticators including depth or thickness of invasion, pattern of invasion, vascular or perineural invasion, bone involvement, presence of therapy-related changes, and preneoplastic lesions. More recently, inflammatory host response, results of molecular studies, and human papillomavirus assays are being collected or clinically requested. The rationale for synoptic reports generated by various professional bodies [11, 12] is to ensure a uniform reporting system encompassing core or mandatory information for diagnosis, prognosis, and treatment of oral cancer as well as gathering complete datasets for tumor registries, clinical protocols, internal quality control, or medical research. In the era electronic medical systems, searchable fields in the electronic pathology database are invaluable for gathering essential information for retrospective studies and clinical protocols. Not all the above data points are considered mandatory, and cancer synoptic reports should be adjusted to local practice since guidelines are regularly updated with the knowledge change. However, presence of the required data elements in the pathology report is currently audited by various regulatory bodies (Commission on Cancer of the American College of Surgeons in United States, Royal College of Pathologists in Great Britain) as performance indicators. Various aspects of the synoptic reporting elements, their clinical import, suggestions for uniform and consistent evaluation, and practical considerations will be discussed below and are listed in Table 5.1. Despite progress in our molecular-genetic

Table 5.1 Elements to be incorporated in oral cancer pathology synoptic report

Clinical elements	Gross pathologic evaluation	Histologic elements	Nodal disease	Other
Demographic data	Specimen size[a]	**Histologic type**	**Type of procedure/dissection**	Inflammation
Procedure	Specimen integrity	**Histologic grade**	**Evaluated lymph nodes number**	Dysplasia present
Specimen type	Specimen fresh/fixed	Depth of invasion/thickness[b]	**Involved lymph nodes number**	Stromal response
Clinical and pathology history	**Tumor size**	Pattern of invasion[b]	**Size of largest positive focus**	Ancillary studies (molecular/immunohistochemistry)
Tumor site	Macroscopic extent of tumor	Microscopic tumor extent	**Extracapsular extension**	Colonization (bacterial/fungal)
Laterality	Gross pattern of growth	**Perineural spread**	Tumor present in soft tissue[b]	**Pathologic TNM**
Clinical TNM		**Lymphovascular invasion**		
		Bone invasion		
		Margin clearance		
		Dysplasia present[b]		
		HPV status in oropharynx[b]		
		Therapy related changes		

Bold indicates mandatory elements for both College of American Pathologists (CAP) [11] and Royal College of Pathologists (RCP) [12] guidelines

[a]Mandatory in CAP synoptic report

[b]Mandatory in RCP dataset

understanding of the disease, histopathologic evaluation remains the gold standard for diagnosis, staging, and prognostication of oral cancer.

The vast majority of oral cancers are squamous cell carcinomas, and of these, lip and oral tongue tumors are preventable [13]. The discussion in this chapter will be limited to this most common oral mucosal cancer: its variants, precursors, and histopathologic parameters that can predict its biologic behavior and assist tailoring management, and a review of potential diagnostic challenges encountered in clinical practice will follow.

5.1.2 Anatomic Considerations

Oral cavity represents a relatively large region bounded by the skin anterior to the vermilion and by the oropharynx posteriorly. It encompasses the mucosal lips, buccal mucosa, floor of mouth, oral tongue, hard palate, maxillary and mandibular alveolar gingiva, and retromolar trigone [14, 15]. The oropharynx is the anterior aspect of the pharynx, encompassing the base of tongue (including posterior third of the lateral aspect), soft palate including uvula, tonsils, tonsillar pillars and fossae, glossotonsillar sulci, and lateral pharyngeal wall. The demarcation of oral cavity from oropharynx is in some areas difficult or even arbitrary, and a large body of literature uses a joint designation (e.g., oral-oropharyngeal) referring to tumors from both sites, including the last WHO classification. In this text, the main discussion will be limited to oral cavity tumors since we believe the tumors involving the two sites should be separated for two main reasons: first, embryologic origin of oral tongue and base of tongue mucosa is different; second, tumors involving oral tongue are different for the most part from those involving the base of tongue and other oropharyngeal sites. Current guidelines are also reflecting the distinction [11, 10], and a different staging system is used by AJCC [15]. A caveat is that some of these borders are relatively thin (circumvallate papillae dividing the oral tongue from the base of tongue, soft to hard palate demarcation) and tumors not uncommonly straddle them. However, it is important for both the pathologist and the clinician to be aware of the current epidemiologic shifting trend from tobacco-related to viral-associated neoplasms and their respective anatomic localization (oral and oropharyngeal, respectively; see discussion below). Oral cancer has decreased and pharyngeal cancer has increased in the last decades in the United States [16, 17] likely due to decrease in smoking prevalence and raising incidence of oropharyngeal human papillomavirus (HPV)-associated tumors. HPV-related oropharyngeal carcinomas are biologically different from the (usually) tobacco-related conventional SCC, which account for the vast majority of cancer involving oral sites. Precise clinical designation of the tumor site, or main tumor bulk origin clinical impression, is important for accurate reporting, staging, and workup by the pathologist. In large tumors straddling two sites or subsites, the center of the tumor is designated by convention the primary site [18].

5.1.3 Sampling Considerations

Both clinical and pathologic samplings are preanalytical variables that profoundly affect the ability of the pathologist to render a correct diagnosis. Due to the inherently limited tissue in any given biopsy, uniform correct sampling is essential to capture the "worst" area of the lesion. Clinical experience and judgment of the operator in making that assessment are critical. Tissue orientation in the paraffin block can affect the ability of the pathologist to assess the presence or depth of stromal invasion and therefore confirm the presence of invasive carcinoma. While no single approach is perfect, based on our experience and published guidelines [10, 11], the following general principles are suggested for the best diagnostic outcome.

Regarding *small samples*, incisional biopsies should be performed perpendicularly to the mucosal plane to the deepest possible point. Punch biopsies (3–5 mm in diameter) are routinely done in our clinical practice with excellent results. Since incisional biopsies represent a limited sample, no evaluation of the tumor borders is possible, and therefore, addressing "margin" status in a biopsy pathology report is confusing, may be misleading or falsely reassuring, and is therefore not indicated. The exceptions are excisional biopsies of small lesions, where completeness of removal may be documented. Given the different reporting requirements, correct clinical designation of the procedure (incisional vs. excisional) is warranted.

A biopsy of the center of the ulcer or a necrotic (friable-appearing) area may result in a nondiagnostic sample (no viable tissue present) and should be avoided. A biopsy of the tumor periphery, ideally sampling the adjacent nonneoplastic mucosa, but deep enough to assure representation of the infiltrating deep tumor front, is a superior strategy. Depending on the size, shape, and localization of the lesion, multiple biopsies can be considered especially in large heterogeneous plaques.

Superficial biopsies horizontal to the mucosal plane (so-called *shave* or *en face* biopsies) should be avoided when possible since many oral lesions are hyperplastic and/or hyperkeratotic, and examining the superficial aspect of the lesion may underestimate its biologic potential. For instance, bland-appearing hyperkeratosis, a common histologic finding in and explanation for the macroscopic white appearance of leukoplakia, may overlie well-differentiated infiltrating squamous cell carcinoma as well as frictional keratosis (Fig. 5.1).

Generally speaking, while important information can be detected in any incisional biopsy and should be documented in the pathology report (perineural or lymphovascular involvement, extension into skeletal muscle) when present, depth of involvement cannot be accurately estimated in most bioptic samples. The inherently incomplete information is not sufficient for issuing a synoptic report [11] for oral mucosal epithelial tumors.

Excisional biopsies, *resections, and neck dissections* should always be oriented by the surgeon since the normal anatomic landmarks are either not present or are extremely difficult or impossible to reconstruct *ex vivo*. If no orientation was received, pathologist's communication with the surgeon is essential.

Adequate tumor sampling in the pathology department is at least one section/cm for neoplastic processes. We routinely examine small tumors entirely and sample much more from larger tumors after tissue procurement.

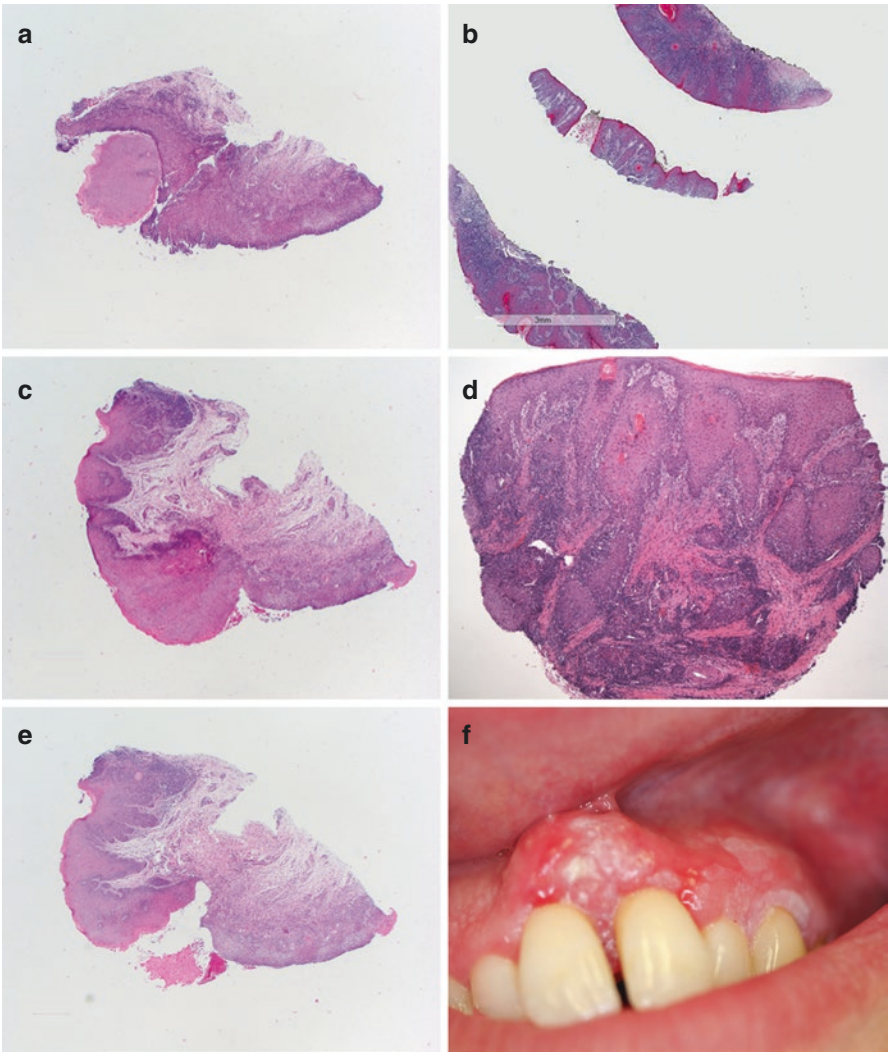

Fig. 5.1 Sampling error may be due to insufficient tissue examined by the pathologist or due to a limited biopsy by the clinician. Step sections of a punch biopsy of an erosive plaque on the lateral tongue of a 39-year-old woman highlight the dramatic difference between first tissue profile (**a**) with completely eroded mucosa and deeper sections (**c**, **e**) revealing viable mucosa with a microinvasive focus of cancer (*left upper* corner). (**b**) A shave biopsy of the gingival nodular erythroleukoplakia in a 43-year-old nonsmoker woman, clinically suspected for carcinoma, was interpreted by two expert pathologists as lichenoid inflammation and pseudoepitheliomatous hyperplasia. (**d**) Subsequent punch biopsy confirmed the presence of well-differentiated squamous cell carcinoma. (**f**) The clinical image strongly suggesting a malignant process was not provided to the consultant pathologists

No lymph node dissection requirements for a minimum number of lymph node yield currently exist but should be mandated in practice similar to other organ systems (e.g., colon). The importance of dissected node number is discussed below.

Pathologists should address the margin status if a malignant tumor is present and should issue a synoptic report with the key elements listed in Table 5.1.

5.2 Squamous Cell Carcinoma of the Oral Cavity

Oral SCCs represent over 90% of oral cancers [19, 20, 21], and the most common histologic type is conventional (or keratinizing) squamous cell carcinoma.

5.2.1 Clinical Appearance and Microscopic Correlation

Most invasive tumors are easily recognizable as cancer: they can be exophytic, polypoid, endophytic, ulcerated, fungating, indurated, or with a fistula-like appearance (Fig. 5.2). Verrucous cancer has a distinctive, cauliflower-like, corrugated appearance clinically but at an early stage is indistinguishable from verrucous hyperplasia. Unlike salivary gland tumors or lymphomas which present primarily as submucosal lesions, oral squamous cell carcinoma usually involves the mucosal surface. An indurated ulcer, the classical presentation of OSCC, was present in only 12% of oral cancers in a recent study [30]. Predominant submucosal growth is relatively uncommon for oral squamous cell carcinoma, in contrast to pharyngeal or hypopharyngeal primary mucosal neoplasms; however, it is not uncommon to underestimate the depth and extent of disease by gross examination only. HPV-associated neoplasms of base of tongue or tonsil are not uncommonly difficult to detect clinically, radiologically, and at pathologic gross evaluation, can be easily missed by a superficial biopsy and only rarely extend to an adjacent oral site such as oral tongue or retromolar trigone.

One of the most commonly encountered lesions is a plaque, either leukoplakia or erythroplakia. The latter has a higher risk of harboring a malignant or high-risk potentially malignant lesion. A biopsy is warranted for prompt diagnosis and management in both cases. Leuko-/erythroplakias, their various appearance and potential for transformation, will be discussed below under "Potential Malignant Disorders" section. The historical prevalence of carcinoma in leukoplakia is 3% [23], but for early cancer, leukoplakia is the most common clinical presentation [24, 25]. The dentists are central in detecting malignant lesions at their earliest, curable stage, and importance of a thorough examination of the entire oral cavity including lateral and ventral tongue and retromolar trigone cannot be overemphasized [20, 24, 26, 27].

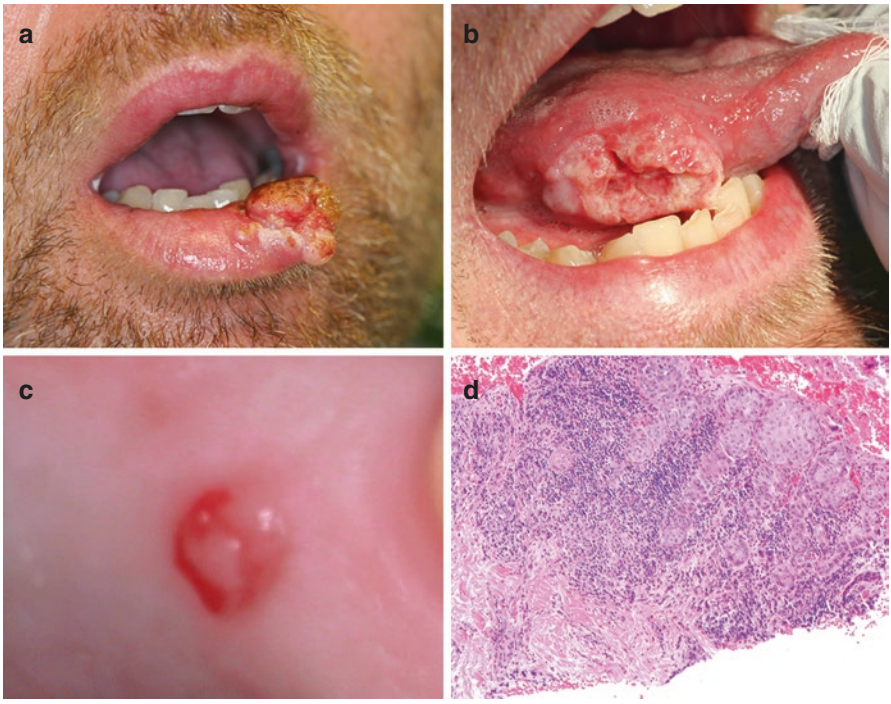

Fig. 5.2 (**a**) This rapidly growing lower lip squamous cell carcinoma of a 39-year-old man with history of bone marrow transplant for acute leukemia developed metastasis in the submandibular region 4 months after excision of the tumor. (**b**) Ulcerated, indurated right oral tongue carcinoma in a 65-year-old man has residual leukoplakia at its posterior aspect. (**c**) Right hard palate erythroplakia in a 54-year-old man was persistent for a month, and its biopsy showed microinvasive carcinoma (**d**) in a background of high-grade dysplasia

Studies assessing the gross appearance-microscopic correlation of tumors are rare. Generally speaking, tumors with predominant exophytic component such as verrucous or papillary carcinoma have a more indolent biology and better outcome than endophytic, indurated, submucosal, or deeply infiltrating tumors.

5.2.2 Diagnostic Pitfalls and Potential Issues

The key for a definitive and timely diagnosis is an adequate biopsy in the situations when clinically indicated, which requires clinical judgment and experience. In large lesions, the decision to biopsy is automatic and pathologic diagnosis usually straightforward. A small lesion should not deter the clinician from biopsy; that decision may be lifesaving since early tumors can be asymptomatic. In fact, detecting small cancers, either isolated or identified from white plaques, may pose significant challenges since over two-thirds are asymptomatic [24, 26, 28, 29]. Moreover, non-ulcerated lesions are often malignant or not suspected clinically about one-third of the time

[22, 30], regardless of the endo- or exophytic pattern of tumor [22, 30]. Delaying diagnosis may deprive the patient of curative management options, and unfortunately, despite easy accessibility, well-known risk factors, well-recognized premalignant lesions, and various adjuvant methods available, no significant progress has been made in detecting early oral cancer in the last decades [20, 26]. Both clinicians and pathologists can improve this state of affairs, increasing detection of incipient cancer, and thus improve survival in these patients. This is particularly important since current evidence was recently deemed insufficient to support screening for the disease by US Preventive Services Task Force [31]. American Cancer Society recommends oral examination as part of the general checkup for cancer in patients over 20 [32] and American Dental Association recommends that health-care providers search for potentially malignant disorders or early-stage cancer during routine oral exam, especially in high-risk patients (tobacco or heavy alcohol users) [33].

For the clinical examination to increase early detection, the reader is referred to Chap. 9 and other textbooks and practice guidelines [34, 35]. Clinicians (surgeons or dentists) should perform a thorough oral examination of the lips, buccal mucosa, and ventral and lateral tongue as previously described, maintain a high index of suspicion and biopsy suspicious lesions persistent after 2 weeks [35] in high-risk areas, and in patients deemed at high risk (significant smoking and alcohol use history). Ulceration of oral mucosal lesions should raise suspicion for OSCC although obviously many nonneoplastic diseases can show erosion or ulceration, and, in a recent series, most OSCCs were not ulcerated [22, 30].

An incisional or punch biopsy, adequate both as depth and width, should be obtained perpendicular to the mucosal plane whenever possible. If the pathology result is negative, but the clinical suspicion is high, then rebiopsy is recommended [26] since precursor lesions are heterogeneous [36].

In the pathology department, the sample should be carefully grossed and processed. Any 5 mm diameter punch biopsy could potentially render approximately 1,000 tissue sections (5 mm = 5 μm/each section × 1,000); therefore, a large area of mucosa may not be represented on the slides. Since various profiles can show widely variable appearance, including small foci of cancer (Fig. 5.1), liberal sampling is recommended to ensure representative sampling without paraffin block exhaustion. Levels at 100 μm have been proposed [10], but each laboratory should set its own standard histologic protocols.

Currently the tumor gross (macroscopic) pattern of growth is not considered an essential data point for the cancer synopsis [11, 12]; however, important clinical information can be extracted from it, and accurate documentation in both clinical and pathologic records is highly desirable.

5.2.3 Oral Cavity Subsite-Related Tumor Characteristics

There is a rich and often conflicting literature on the topic of subsite heterogeneity of oral cancer and its clinical and biologic significance. It was recognized for almost half of century that significant differences exist between squamous cancer of various oral subsites [29]. One earlier proposed modification of the TNM system

included both oral subsites and pathologic characterization of the lesion – STNMP (Site, TNM, Pathology) [37]. While this proposal did not gain acceptance, the reader will note in the following text that many site-specific features, several histopathologic features (e.g., perineural spread), and tumor subtype (e.g., verrucous carcinoma) originally included were subsequently confirmed and some remain critical in current clinical management decision.

Various subsites of oral cavity may show different clinical characteristics, evolution, and biology: posterior tumors are performing worse [38], and certain protein expression patterns vary in both normal and neoplastic tissues between various subsites [39]. Tumors from various subsites may also have different metastatic potential [40].

Approximately 80 % of oral squamous cell carcinomas arise from lateral and ventral oral tongue, floor of mouth, and soft palate complex, a triangle classically designated as "high-risk areas," comprising only 20 % of oral mucosa surface. The high incidence of cancer surrounding Wharton's duct was originally attributed to various carcinogen pooling (draining), nicotine included [24, 28, 41].

On the other side, lip cancer is likely more akin to cutaneous squamous cell carcinoma arising in the skin with actinic damage, and this is probably why it has the best survival, in addition to earlier detection. Conversely, posterior tumors fare worse than anterior ones, possibly due to more advanced stage at presentation and/or surgical difficulty for complete removal [38].

Tumors involving specific oral subsites may be different due to histoanatomic variation including variable local vascularity, type of oral mucosa – thin, nonkeratinized in floor of mouth and lateral-ventral tongue and thick, keratinized in masticatory areas, density and thickness of submucosa, and subjacent structures such as muscle or bone which may represent a natural barrier to the disease; clinical aspects (ease of detection, stage at detection, and surgical accessibility); and intrinsic tumor genotype-phenotype and biologic characteristics. Unfortunately, review of the literature is problematic since various tumors with different histologic subtypes, clinical stage, and location are often grouped under the "oral cavity" anatomic umbrella in most large studies.

Some practical aspects of difference in carcinogenesis, clinical-histologic features impacting management, and prognosis of oral squamous cell carcinoma involving different subsites will be summarized below.

Mucosal lip carcinoma involves the areas posterior to the skin border, including the vermilion and commissures but restricted to mucosal lining in contact with the opposing lip [15]. It is one of the most common sites of oral carcinoma, with the lower lip accounting for >90 % tumors [13]. There are both geographic (in Australia lip cancer accounts for over 50 % of oral cancer [42]) and race differences, only 1.1 % of patients being African-American. It is commonly a disease of white men except for upper lip tumors which are more frequent in women [19]. Clinically, they are variable in appearance, usually involving the vermilion between commissure and midline (Fig. 5.2) [19].

Histologically, most tumors originating at this site are predominantly well and less frequent moderately differentiated conventional (keratinizing) SCC, clinically

similar to primary skin cancer. Most are associated with, and likely caused by, pre-existent actinic damage with typical microscopic changes often present adjacent to tumor (Fig. 5.3).

Several studies reported an impact of tumor grade on patient outcome, unlike other oral subsites [43, 44, 45]. The acantholytic (adenoid, pseudoglandular) variant of SCC, more frequent seen in skin tumors, is a rare variant of oral SCC most commonly encountered in the lip [18] and also usually associated with actinic damage.

Lymph node involvement occurs in 5–12 % of cases [19, 46] although the range in the literature is much broader (3–29 %) [45], usually late in the course of disease and mostly in large or deeply infiltrating tumors. Pattern of invasion and depth of invasion are strong predictors of nodal metastasis [43–47, 49]. One group recently proposed a risk model involving tumor thickness and differentiation with high-risk group including well-differentiated deep tumors (>5 mm tumor depth) and/or more superficial tumors (>2.5 mm depth) with moderate (G2) and poor (G3) differentiation, while the low-risk group include all thin tumors, G1 <5 mm and G2/3 <2.5 mm. Such a model allowing stratification for neck management requires prospective validation and uniform pathologic assessment, and it was suggested that the

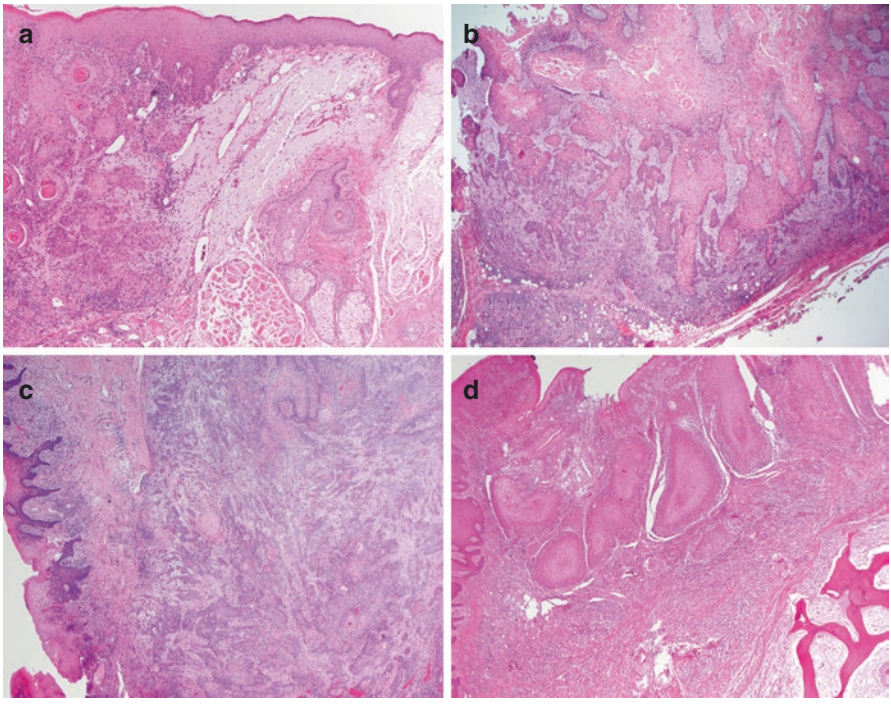

Fig. 5.3 Oral cancer at various subsites. (**a**) Lip carcinoma of the vermillion associated with extensive actinic cheilitis (*right upper*). (**b**) Floor of mouth squamous cell carcinoma involving sublingual salivary gland (*left lower*). (**c**) Deeply infiltrating oral tongue carcinoma with ulceration. (**d**) Alveolar cancer with erosion but not infiltration of maxillary bone cortex (*right lower*)

information could be obtained from a punch biopsy [45]. Caution is essential since earlier experience has shown that punch biopsies may underestimate the depth of invasion in lip cancer >3 mm deep [48].

Lip cancer usually presents at early stage [50] and has the best overall prognosis (over 90 % relative survival at 5 years) of all oral cancers in the United States [21, 50]. Survival was traditionally said to be worse in upper lip tumors [19], but recent data dispute that tenet [45, 51].

In sum, lip squamous cell carcinoma is anatomically, histologically, and biologically at the interface of cutaneous and intraoral carcinoma, probably closer to the former. Most tumors are detected early, are well differentiated, and, if less than 2 mm deep, do not metastasize.

Buccal cancer involves the mucosae of the opposing lips, inner to the line of contact described above, and mucosa of the cheeks bordered by the upper and lower alveolar ridge attachments and overlying the buccinator muscle. Parotid gland opening (Stensen duct) is located at the fold opposite the upper second permanent molar and may be involved by tumor with subsequent obstruction.

Buccal squamous cell carcinoma comprises less than 10 % of oral cancer in the Western countries but accounts for 40–44 % of oral tumors in India, due to betel/tobacco chewing prevalence [13, 19]. A high incidence was reported in the Southern United States where chewing tobacco is common. Due to high incidence of this tumor on the subcontinent, recently Indian guidelines for cancer involving buccal mucosa were published [52]. It is a disease of the elderly in the West, with average age at presentation in the seventh decade [29].

The usual clinical presentation is that of an exophytic tumor, often associated with or surrounded by leukoplakia, but ulcerated or verrucoid tumors, and, rarely, a fistula tract that may involve the entire buccal wall to the skin may be encountered. In the latter, establishing the primary origin (mucosal versus cutaneous) may be challenging, particularly in large tumors.

Histologically, keratinizing squamous cell carcinoma is the most common type, usually moderately differentiated [53, 54], in contrast with lip cancer. Buccal mucosa is one of the most common sites for verrucous carcinoma, arising primarily along the buccal-gingival sulcus. This variant has a superior prognosis and different biology than conventional squamous cell carcinoma (see below under histologic subtypes). Deep infiltration of the buccal fat and buccinator muscle is present even at early stage. In up to half of cases, the tumor approaches the mandibular or maxillary bone requiring bone surgical resection [54, 55]. Once a fistula tract is formed to the skin, recurrences can be difficult to diagnose, particularly in the radiated field (Fig. 5.14).

Histologic adverse prognostic factors, as in other oral subsites, include lymph node involvement and extracapsular extension. Bone involvement and perineural invasion, the latter reported seen in approximately one-third of the cases, are also poor prognosticators [54–56]. Muscle invasion and Stensen's duct involvement were associated with decreased survival [55]. Probably due to its indolent growth, SCC in this location usually presents at advanced stage and is reported to have high rates of locoregional recurrence. Recent data suggested that a 3 mm margin may be

sufficient for complete resection [53], and others found no significantly decreased 5-year survival despite increased percentage of positive margins [54]. Carcinoma of the buccal mucosa was said to be worse than other oral subsites in the older literature, but this was likely due to advanced age and stage at presentation as recently shown in a SEER database review [57] as well as other recent studies [54, 58, 59]. Despite strikingly distinct prevalence and somewhat different carcinogens involved in buccal carcinogenesis on the two continents, similar prognosis was reported on two cohorts from India and Canada after age and site matching [60]. This would argue against an intrinsically more aggressive biology of the tumor at this site. Again, early detection is essential in improving outcome.

In sum, buccal mucosa squamous cell carcinoma is relatively rare in the West, presents at advanced age and stage, but, if detected early, does not portend a worse prognosis compared with other oral subsites.

Carcinoma of gingiva and alveolar mucosa (alveolar ridge cancer) involves the mandibular and maxillary mucosa (lower alveolar and upper alveolar ridges) extending from the gingivobuccal sulci to the floor of mouth and hard palate junction medially, and posteriorly to the ascending ramus of the mandible and pterygopalatine arch, respectively [15]. Although there is an anatomic distinction between gingiva (attached and free) and alveolar mucosa, cancer involving either will be discussed below.

This is the third most common oral cancer after oral tongue and floor of mouth and is associated with tobacco use, snuff dipping, alcohol consumption, and poor oral hygiene. Most commonly tumors involve the premolar/molar aspect of the mandibular alveolar ridge, whereas less than 25 % involve the upper jaw [19, 61]. Interestingly, gingiva is the second most common subsite for pediatric oral squamous cell carcinoma [62].

Gum cancer is the most difficult to diagnose oral cancer [29], both clinically and histologically, particularly at an early stage. It can be associated with leukoplakia or it may present as an erythematous area, but a large ulcerated mass at presentation is relatively rare. A nonhealing, persistent post-extraction wound is a very common presentation in alveolar tumors in our referral center. The particular histoanatomy of oral mucosa lining in this location, lack of significant submucosal soft tissue, and its proximity to the underlying bone influence the pattern of involvement, difficulty in clinical detection, and insidious presentation of these tumors.

Histologically, the lack of submucosa makes the assessment of soft tissue involvement challenging particularly in small lesions or when only a minimal sample is obtained. Depth of invasion cannot be reliably assessed in this location [63]. Most tumors are keratinizing squamous cell carcinoma, and histologic grade was reported to be important in predicting nodal metastasis and survival [64–67]. Dysplasia and microinvasion are often difficult to assess due to the specific histoanatomy of gingival-alveolar mucosa.

Bone proximity results in the propensity of this tumor for bony invasion. Earlier this process was thought to occur through periosteal lymphatics [68], but it has been demonstrated later [69–71] that direct destructive involvement either at the point of tumor abutment in completely dentate patients (usually at the junction of attached

and reflected mucosa) [71] or through occlusal surface in edentulous patients is most common. The frequency of bone invasion in gingival cancer is widely variable in the literature, depending on the definition and methodology employed in various studies, but it has been reported in 39–65 % of cases [65, 67, 72]. The pattern of bone invasion (erosive vs. infiltrative) is important for the prognosis and surgical planning; however, significant variability exists between accuracy of various radiologic methods, correlation between the imaging and histopathologic findings, and type and completeness of histologic assessment from case to case or center to center.

Histologically, there are two main patterns of bone invasion: the *infiltrative* or invasive pattern [73, 74], which is osteoclast independent [69], with tumor cells deeply infiltrating medullary space without intervening soft tissue, and the *erosive* pattern, associated with increased osteoblastic activity and a pushing tumor front separated from the bone by soft tissue and osteoclasts, usually limited to the cortex, or superficially invading the bone (Figs. 5.3 and 5.9). A transition or progression between the erosive to the infiltrative phase (so-called mixed pattern) has also being described [74].

Involvement of the inferior alveolar nerve, believed earlier to constitute an important mechanism of tumor spread into the bone, particularly in the previously irradiated and edentulous mandibles [70], was later proved to be much less important [67, 74] and infrequent in the nonirradiated jaw: in the single study to date where the entire tissue was submitted for histologic examination, perineural invasion was identified in less than 25 % of tumors with deep (alveolar canal) infiltration and in none of the of superficially invading tumors [75].

Majority of the alveolar ridge tumors involved the lower jaw [61], but gingival tumors may involve the maxillary bone. Due to the anatomical contiguity and biologic similarity, these tumors have been often reported together with hard palate tumors – please refer to that section for discussion. The correlation between the presurgical imaging and histopathologic findings is less than ideal for the detection of and distinction between bone erosion and invasion [71, 72, 75, 76]. Computed tomography is the usual preferred test, but magnetic resonance imaging was suggested for its higher sensitivity [71]. Histologic examination remains the gold standard for evaluating bone invasion, its pattern, and depth. Of note, complete histologic assessment is not always feasible or performed in routine practice (i.e., microscopic examination of the entire bone tissue), and therefore, it is subject to sampling artifact. A number of medullary space or perineural invading microscopic foci of tumor may not be sampled. It has been suggested that intra- or perioperative periosteal stripping tissue evaluation may guide the necessity for segmental mandibulectomy [77].

The controversy on adequacy of marginal mandibulectomy in oral cancer continues, primarily due to the variable clinical and radiologic techniques utilized in clinical practice preoperative evaluation and definition of bone involvement. In a recent review, gross bone involvement in the previously irradiated jaws and clear-cut medullary space involvement radiologically were considered the only indication for segmental resection [78]. Bone and soft tissue margins are important and will be discussed later (see prognosticators).

Reported adverse prognostic indicators of gingival carcinoma include higher clinical stage [61, 65], nodal involvement, previous dental extraction [61], moderate to poorly differentiated histology [65], and pattern of infiltration in the soft tissue [71].

In sum, gingival carcinoma can be difficult to diagnose and presence of bone involvement challenging to ascertain before surgery. Edentulous patients have a higher propensity for occlusal involvement, but most tumors involve through direct extension into the bone. Histologic pattern of invasion (erosive versus infiltrative) may guide the appropriate surgery and prognosis. Clinical detection of gingival cancer before invading the bone or with exclusively erosive pattern of superficial (periosteal/cortical) involvement will undoubtedly improve the overall outcome.

Retromolar trigone cancer (*RMT*) involves mucosa attached to the ascending mandibular ramus posterior to the last molar and bordered medially by the anterior tonsillar pillar, floor of mouth, and soft palate. Retromolar trigone is an area of oral cavity [14, 15], and these tumors, among the least common of all oral subsites [79], are associated with tobacco and alcohol abuse. RMT tumors are not uncommonly misassigned to the oropharynx in clinical practice. Some authors classify the RMT tumors under the oropharynx, based on purported similar behavior with primary oropharyngeal tumors [19]. Most authorities, however, indicate that RMT tumors are similar to alveolar ridge carcinoma [80, 81]. It is important to correctly distinguish RMT from the oropharyngeal tumors since individual surgeons and institution philosophy may be vastly different in managing the two [82, 83]. Adjacent sites are in close proximity, but the UICC tumor epicenter rule should be applied, and primary site should be reassigned to the buccal, oropharyngeal, floor of mouth, or alveolar gingival subsites if retromolar trigone is only secondarily involved. In some cases, the distinction may be difficult or impossible [84]. Tumors of the retromolar trigone extend to buccal mucosa, oropharynx (anterior tonsillar pillar), mandibular or maxillary bone, and due to their posterior localization, high stage at presentation and high recurrence rate, require aggressive multimodality therapy [82, 83, 85].

Similar to alveolar ridge cancer, both mandibular and maxillary bone can be involved in 11–15 % of RMT cancer [82, 86], but recently rates of up to 34 % were reported [88]. Tumor spread into the cervical nodes at levels I and II is clinically detected at presentation in 27–60 % of cases [19]. One oft-cited study reported 64 % occult neck metastases and 78 % total positive lymph nodes; however, this cohort was relatively small and included anterior tonsillar pillar tumors, which may be a confounding factor [84]. All patients with bone involvement require surgery; when surgical margins are involved, survival was nil in one series [87]. Other adverse prognostic factors are masticatory space involvement and neck recurrence [88].

Histologically, most tumors are similar to the gingival cancer and are conventional keratinizing squamous cell carcinoma, but the literature on the specific pathologic features is sparse.

In summary, retromolar trigon SCC studies are few and results widely variable due to anatomic classification challenges and probably referral bias. No pathologic oriented study of these aggressive tumors has been reported to date.

*Hard palate carcinoma i*nvolves the area of the roof of the mouth, a semilunar area bordered by the upper alveolar ridge, and mucosal surface covering the palatine process of maxillary palatine bones. It continues posteriorly with the soft palate, which is a part of the oropharynx, and medially with upper alveolar ridge mucosa, transition being gradual and the demarcation between the two subsites ill defined. For this reason, many studies combine the hard palate and upper alveolar tumors [89–92, 97].

Hard palate is the least common subsite for intraoral squamous cell carcinoma but the most common for minor salivary gland carcinoma. It is usually a disease of the elderly occurring in the seventh decade [92]; however, unlike other oral sites, there is a slight female predominance [92, 93, 96]. Clinical presentation is usually leukoplakia [19], but in about a quarter of the cases, ulceration or exophytic lesions are seen.

Histologic type of the hard palate squamous cell carcinoma is well differentiated in most cases [92]. Bone invasion is variable, but most cases present at an advanced pathologic stage regardless of geographic region; however, the rate of pT4 disease is much higher in Southeast Asia [95] compared to Western studies [92, 94]. Locally advanced tumors involve the maxillary sinus, nasal cavity, gingiva, and soft palate [19]; involvement of the latter was associated with a worse prognosis [98].

Higher rates of positive margins are historically known to be associated with hard palate and upper gingiva [99] compared to other intraoral cavity sites. This trend has been persistent in recent studies as well [89, 93]. Neck dissemination involves the submandibular and subdigastric stations [92]. Recent studies revealed a regional recurrence rate in the 26–28 % range [92, 93], strikingly similar to the rate of occult node involvement reported by a different group [94].

Poorly differentiated histology impacted survival in several studies [94, 97] but not in others [92]. Elective neck dissection may be considered for stages > T1 due to relatively higher rate of locoregional recurrence compared with other oral sites [91, 92, 93, 94] and relatively comparable rate of occult neck metastasis with other sites.

In summary, hard palate and upper alveolar squamous cell carcinoma share biologic similarity and anatomic proximity, and clinically are managed similarly. An increased risk for positive margin compared to other sites and higher metastatic potential than previously thought were recently reported suggesting that tumors of these sites may need more aggressive therapy and neck management.

Floor of mouth carcinoma involves the U-shaped, semilunar area bordered by ventral tongue posteriorly and lower gingiva anteriorly and laterally. It is separated by the midline frenulum and has the duct opening of submandibular and sublingual glands [15]. Floor of mouth (FOM) is covered by thin, lining-type squamous mucosa without keratinization and, unlike oral tongue, lacks a dense skeletal muscle layer underneath.

As previously alluded, FOM is considered a high-risk area for squamous cell carcinoma development and is the second most common oral cavity site after oral tongue affecting elderly man with history of smoking. Of all oral subsites, FOM cancer has the strongest association with smoking [24, 100, 101]. Most commonly

involved is the anterior compartment adjacent to the lingual frenulum [28, 80]. It is a common site for erythroplakia, and about half of these lesions are invasive carcinoma [102]. Floor of mouth tumors extend to underlying salivary gland and lingual and mental nerves. Underestimating the depth and extent of involvement is not uncommon, may lead to delay of diagnosis [103] and may be the underlying reason for the increase rate of positive margins in tumors at this site [104–107], recently confirmed in a large retrospective national cancer database analysis [108]. Even mild or moderate dysplasia when present at the margin is increased with high recurrence rate [109].

FOM SCC is often associated with concurrent involvement of lateral tongue and in large tumors the epicenter may be difficult to establish. Many centers and numerous studies do not separate between floor of mouth and oral tongue cancer which are jointly reported. Several histoanatomic differences do exist which will be discussed below.

Most FOM tumors already extend beyond the confines of the site at the time of diagnosis [80] through soft tissue spread: mylohyoid muscle and lingual or mental nerves. Salivary glands are often involved (Fig. 5.3). Perineural involvement is an adverse prognostic indicator and may play a role in bone invasion particularly in edentulous and irradiated patients [70, 80]. Bone invasion occurs in 15–29 % of cases, dependent on tumor proximity to the mandible [80], usually through direct bone involvement as previously discussed [71].

Due to common involvement and crossing of the midline aspect of floor of mouth, bilateral neck dissection may be required [356]. Involvement of the Wharton's duct may generate obstructive symptoms. Histologic distinction of intraductal extension of dysplastic intraepithelial lesions from infiltrative carcinoma may be challenging for the pathologist but is of outmost importance (Figs. 5.3, 5.7 and 5.20).

Tumor thickness is a critical prognostic indicator for early FOM cancer. FOM tumors can metastasize when thickness is less than 2 mm, whereas in oral tongue that threshold is 4 mm [114–117]; others recently reported opposite results [118]. FOM has the thinnest oral cavity mucosa measuring approximately one-third of the buccal mucosa thickness [119].

Most floor of mouth SCCs are moderately and well differentiated, therefore histologic grade has questionable, if any, prognostic value: some investigators found predicting value [108, 118, 120], while others did not [121, 122]. The pattern of invasion is an important prognostic factor for neck involvement and disease-specific survival which has been confirmed by multiple groups [110–113, 118, 122]. It has been shown that floor of mouth tumors are more aggressive and can metastasize earlier than other oral site cancers [110, 117, 120, 123].

Occult nodal disease is present in 21–23 % of FOM SCC stages I–II [105, 116, 124], levels I and II are most commonly involved, found in 30 % of FOM SCC patients at presentation [80, 125], but in tumors of all stages, nodes are involved in 39–45 % of patients [103, 120]. Bilateral sentinel node biopsy may be an alternative to bilateral neck dissections for the midline tumors [126].

In summary, floor of mouth squamous cell carcinoma is an aggressive tumor with propensity for neck metastasis. Pattern of invasion and tumor thickness/depth

are the most important histopathologic predictors for neck involvement and survival.

Oral tongue cancer involves the mobile anterior two-thirds of ventral and lateral tongue including the tip, the lateral surface, and the dorsal aspect. The base of tongue, the part of the organ posterior to sulcus terminalis, belongs to the oropharynx.

Oral tongue squamous cell carcinoma is a disease that occurs in the elderly (median age 60 years), smoking and alcohol being the main associated etiologic factors in the Western world. Its incidence in younger adults (less than 40 years old) has increased from 3 to 6–7 % of all oral tongue carcinoma in recent decades [19]. These patients are usually nonsmokers, attempts to identify a viral etiology were unsuccessful, and they may have a more aggressive course than older patients (see below).

Lateral-ventral tongue is the most common primary site for intraoral cancer [21]. Lateral tongue is also a common site for preneoplastic plaque lesions, and early carcinoma can be detected in potentially malignant disorders. Thorough oral examination and aggressive biopsy philosophy may allow detection of early disease in this high-risk subsite. Tumors of the mobile tongue present typically as an indurated ulcer, an exophytic mass (Fig. 5.2), but occasionally are detected at an early stage in leukoplakia specimens submitted for histological evaluation. Oral tongue was reported to be the most common site for microinvasive carcinoma, and therefore adequate sampling and/or excision of high-risk leukoplakia is essential for early detection (Figs. 5.1, 5.4 and 5.17) [24].

Unlike cancer in other subsites with posterior topography (e.g., retromolar trigone) or sites associated with subtle findings (e.g., alveolar ridge and gingiva) which may require a complex surgical approach, oral tongue is easily accessible for self assessment and examination, and small tumors can be readily excised. It is therefore somewhat surprising that the rate of positive margins and local recurrence rate were 18 % and 22 %, respectively, in experienced hands [127] even in stage I tumors [128]. Recurrence through the deep soft tissue positive margins was suggested to play a significant role by several investigators [129, 258]. It may be clinically challenging to accurately assess preoperatively the depth and extent of muscle involvement particularly in tumors with aggressive pattern of growth (Figs. 5.7 and 5.8).

The histologic grade can be generally predicted with fair accuracy from the gross appearance: exophytic tumors are predominantly low histologic grade, whereas the ulcerated and endophytic tumors are associated with a higher-risk histology [127]. Planning a wider surgical resection in indurated/ulcerated lingual tumors where feasible may be considered [130].

The two most important pathologic predictors of neck metastasis in lingual cancer are the pattern of invasion and tumor thickness. It has been known for over four decades that the pattern of tumor invasiveness, particularly at the tumor-soft tissue interface, has a strong impact on the disease-specific and overall survival and is a predictor of likelihood of cervical lymph nodes spread [104, 118, 122, 127, 128, 131–135, 138–140] with only rare contrary reports the exception [121]. Recently, a modified system built on previous iterations included the

pattern of invasion (POI) scores (1–5), lymphoid host response and perineural invasion was dubbed histologic risk assessment (HRA) [122], and was recently validated in a multicenter study [137].

Tumor thickness is another important predictor of neck metastasis of oral tongue cancer and will be discussed in detail below; however, a 4–5 mm cutoff became currently accepted and recently proposed to be integrated in a new staging system for all head and neck carcinoma [141, 142].

Perineural invasion occurs in approximately one-third of the patients, is associated with large tumor size, infiltrating pattern of growth, tumor thickness, and positive surgical margins, is a predictor of neck involvement, locoregional recurrence [143], and decreased disease-specific and overall survival [56, 104, 144–146, 147]. Recently, a prognostic distinction was suggested between involvement of small nerves (<1 mm diameter) and large nerves (1 mm or more in diameter) [122]. Perineural spread is not uniformly defined, but when a larger (named) nerve is involved, it may be detected clinically or radiologically [147]. There is recent evidence that perineural invasion significance is lower when found intratumoral compared to outside tumor outline [148].

In sum, oral tongue squamous cell carcinoma is an aggressive disease with a high propensity for local recurrence and neck metastasis particularly in patients with perineural invasion, aggressive pattern of invasion, and thickness of 4–5 mm or more.

5.2.4 Pathologic Prognosticators of Oral Squamous Cell Carcinoma

It is well recognized that the current oral cancer staging system has limitations in stratifying the patients at higher risk for developing locoregional recurrences, distant metastasis, and therapy failure. Current progress and refined diagnostic and management strategies for oral cancer treated in specialized centers yielded better outcomes than the historical overall survival of about 50% at 5 years, and therefore reason for optimism exists [149]. However, many tumors are still detected at a late stage when the prognosis is often guarded. With a thorough clinical evaluation and optimal sampling, dentists, surgeons, radiologists, and pathologists are all essential in accurately staging and diagnosing oral cancer at its earliest. Prospective collaborative efforts to solve unsettled issues with the following prognosticators are needed; some were recently addressed in multiauthor review papers on critical pathology parameters including surgical resection margins and evaluation of neck specimens [150, 151].

Several prognostic indicators have been consistently demonstrated to correlate with disease-specific survival, local and regional recurrence, and lymph node metastasis in numerous single or multicenter studies, mostly retrospective. The strength of association of different histologic parameters with the clinical outcome, tumor biology, and its progression has been widely variable, likely due to different cohorts studied and various end points, but, most importantly, probably related to different definitions, classification system, and measurement methods employed.

It is clear that many associations between various parameters (e.g., perineural invasion, pattern of invasion) are measurements of intrinsic tumor aggressiveness, whereas others are both operator and observer dependent (positive resection margins). Ideally, the respective weight of each histologic parameter in determining outcome would be best gauged in a prospective, multicenter study using a uniform, standardized pathologic examination, assessment, and scoring tumors matched for stage and oral subsite, employing similar management protocols with adequately powered cohorts. No such study exists to date and consequently similar studies have reported conflicting results. However, a recent trend for multicenter collaborative efforts seemed to expand from the clinician to the pathologist investigators [152]. Such an approach will ensure method validation and consensus building and provide sufficient statistical power required for definitive answers for the critical clinical questions currently still under debate.

5.2.4.1 Tumor Site

Primary site of involvement is a well-recognized prognostic factor as previously discussed: the more posterior the tumor, the higher the likelihood for nodal metastasis [38, 153]. There are also geographic and, probably related, etiologic differences between site significance of oral cancer in Western and Asian patients, as shown by a recent large Sri Lankan study in which retromolar trigon had the smallest potential for metastasis and the palate cancers were the worse, although staging data was not available [40].

5.2.4.2 Tumor Size

Larger tumors are associated with higher recurrence rate, nodal metastasis, and poor survival [46, 55, 67, 86, 104, 106, 113, 155–159]. However, for small, superficial, or broad tumors, this correlation is much weaker or becomes insignificant since the volume of the tumor is less predictive than its thickness [160]. Usually, the largest tumor diameter is measured parallel to and its thickness or depth is measured perpendicularly to the mucosal plane. Tumor size is a mandatory data point for cancer synoptic reporting.

5.2.4.3 Tumor Thickness (TT) and Depth of Invasion (DOI)

TT and DOI are major prognosticators for OSCC. It has been generally accepted for a long time that exophytic tumors (verrucous carcinomas being the prototype) or predominantly exophytic tumors have a good prognosis, whereas endophytic or deeply infiltrating tumors are aggressive [161]. Tumor thickness (TT) is an important independent factor in prognosis with strong effect on disease-free survival and overall survival, correlating with propensity for nodal spread better than tumor size in oral cancer. This was shown both in now classical studies [104, 114, 115, 161, 162] and in more recent ones [113, 155, 160, 163–165]. Negative studies are the exception [166]. A floor of mouth 0.9 cm wide tumor infiltrating at 0.7 cm depth will likely have a worse outcome and carries a higher risk of neck metastasis than a 1.9 cm wide tumor with microinvasion or superficial invasion (e.g., less than 2 mm in thickness) [160]. This understanding has been shared by

both pathologists and surgeons for a long time and is used in clinical practice. Introducing these measurements as part of oral cancer staging has been proposed by several groups in the last 30 years [115, 142, 144, 160, 161, 168, 170–172]. It is therefore puzzling that a uniform specific measurement definition lacks a consensus yet and even widely used textbooks by all oncologic clinicians such as AJCC Manual [15] still present various options of measurement. A direct consequence is that the proposed critical cutoff value for elective neck dissection or radiotherapy is variable.

Several reference points were variably employed in the literature, as thoroughly analyzed in two excellent reviews [141, 167]. The deepest point of invasion is the only constant, uniformly accepted and used reference point in all studies, an imaginary horizontal line, parallel with the surface mucosa, drawn through the level of deeper most tumor cell cluster. The second point of reference to determine depth or thickness is extremely variable, controversial, and lacking consensus at this time. Various authors used different superior reference points for their measurements (Figs. 5.4 and 5.5): surface of tumor, surface of normal adjacent mucosa, base of normal adjacent mucosa, and ulcer base/bed in ulcerated tumors [11, 15, 141, 167]. These differences may account for different, at times conflicting, results reported in many studies analyzing similar cohorts.

Tumor thickness is defined simply as the largest vertical dimension between the surface of the tumor (excluding ulcerated areas, keratin and parakeratin) and the deepest point of invasion [11, 104, 161]. TT is deemed the most *objective*

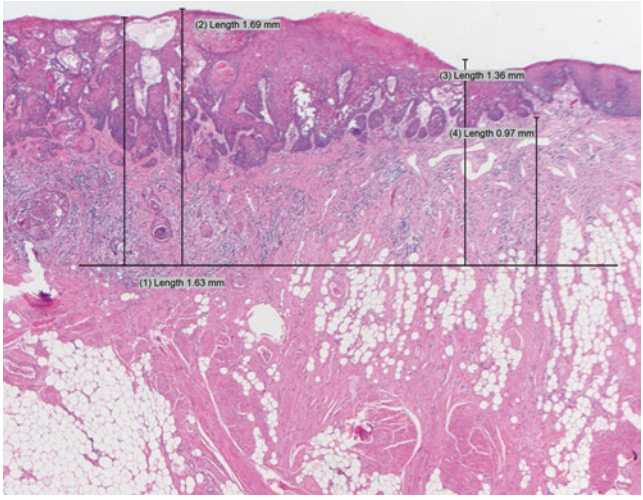

Fig. 5.4 Tumor thickness/depth of invasion (TT/DOI) measurement variability. Various reference points have been used in different studies: from the same deepest point of invasion (*horizontal line*), TT may be perpendicularly measured to tumor surface (*1*) or to the *highest* possible tumor surface point (*2*). DOI can be measured to the adjacent normal mucosa (*right upper* aspect) surface (*3*) or to its basement membrane (*4*). A 0.72 mm (43 %) difference between the highest and lowest possible TT/DOI values may be seen in this superficial tumor

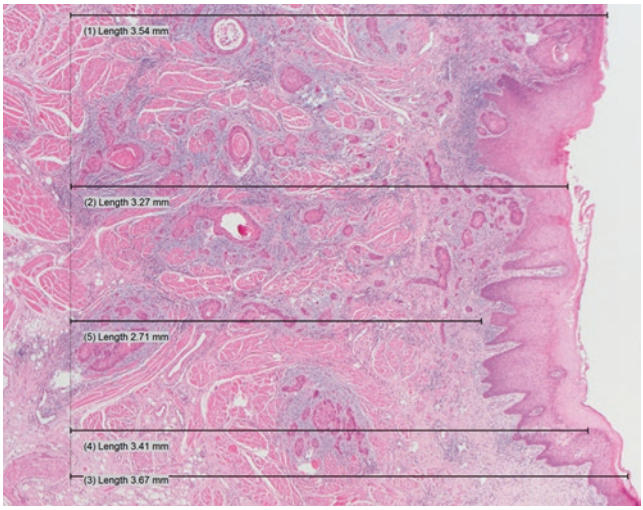

Fig. 5.5 Tumor thickness/depth of invasion (TT/DOI) measurements. In this slightly deeper tumor, TT is measured from the same deepest point of invasion (*vertical line*) perpendicularly to the *highest* possible surface point (*1*) or to nearest tumor surface (*2*). DOI can be measured to the adjacent normal mucosa (*right lower* aspect) surface (*3*) or its basement membrane (*4*). If adjacent normal mucosa is absent, the DOI may be measured from the adjacent hyperplastic/dysplastic mucosa (*5*). A 0.96 mm (26%) difference between the highest and lowest possible TT/DOI values may be clinically significant

measurement, is often the easiest, but tends to overestimate the malignant potential of exclusively or predominantly exophytic tumors. The example of verrucous carcinoma which can be quite thick but never metastasize is illustrative, and it was identified in one of the earliest studies [161]. Therefore, many investigators use as reference a line through the adjacent normal mucosa and not tumor surface (Figs. 5.4 and 5.5).

Depth of invasion (DOI) is measured from the deepest tumor point to the level of *basement membrane* (actual, where there is no ulceration, or virtual, reconstructed in ulcerated tumors [163]) or to an actual or a reconstructed line through the *surface* normal mucosa, adjacent to tumor, similar to Breslow's system (Figs. 5.4 and 5.5) [113]. While some authors [167] suggested that normal epithelium is too thin to significantly affect the thickness and/or depth regardless of the points chosen (surface or base of normal mucosa), it should be considered that many of the early oral tumors (precisely the tumors for which this measurement is most informative) are surrounded by hyperplastic epithelium, which in areas may approach 1 mm in thickness.

In addition, normal mucosa thickness varies within and between subsites. In our hands, the thickest nonneoplastic mucosa adjacent to T1 oral carcinomas ranged from 0.1 to 3.1 (mean 0.8) mm and therefore can significantly affect measurement since mean TT was 4.5 mm in this cohort [169]. In the same study, we have found that even when the measurement tool and strategy are identical, some interobserver

variability exists and that DOI has higher reproducibility than TT (intraclass correlation coefficient of 93 vs. 75) when no common strategy is used by various pathologists (study measurements compared to those recorded in pathology reports) [169]. Figures 5.4 and 5.5 illustrate the differences between measurements that would be rendered using different reference points or measurement strategies in the same tumor when deepest level of invasion is predetermined.

Basement membrane level would be a more logical and biologically significant point of reference even when reconstructed [137, 163, 167, 171] for the simple reason that the tumor component above the basement membrane level (exophytic) theoretically lacks or has limited access to submucosal lymphatics. However, some of the same authors agreeing with this argument [167] deemed TT a more reliable measurement and used it instead of DOI in subsequent studies [24]. As discussed above, this has not been our experience. Other authors clearly describe in the method's section tumor thickness, but they refer to it as "depth of invasion" [139] and the confusion regarding reference points for measurements persists in recent large studies from experienced centers [117, 173]. When tumor is ulcerated, the measurement from the ulcer bed to the deepest point (TT) may be misleading and underestimate the malignant potential since tumor present deep into soft tissue has access to lymphatics. DOI would be most reliable here provided that adjacent normal mucosa is present on the same slide with the deepest point of invasion.

Literature analysis is hampered by small retrospective cohort studies, different tumor stage (T1–4), different outcome points, tumor subsites, variable definition of neck involvement, and, most important, interchangeable usage of depth of involvement and tumor thickness.

A single study comparing DOI in a small cohort of patients with tumors from various oral cavity subsites concluded that depth of invasion has a stronger correlation with neck metastasis than tumor thickness [171]. Several issues need to be considered regarding TT/DOI variability and its practical usage will be addressed below.

(i) *Sampling*. As shown by Breslow in his landmark papers [174, 175], serial sampling of the tumor is essential in identifying its deepest point. In larger tumors, reconstructed basement membrane (or mucosal surface line), a careful sectioning encompassing both the deepest point and the adjacent nonneoplastic epithelium is usually not problematic in T1 lesions; however, if the tumor has a wide surface (>3 cm), it may be difficult to provide adjacent mucosa for reference in ulcerated tumors. More intensive sampling may be helpful in these cases, or one or several full thickness sections with the largest diameter divided in several contiguous sections may allow visualization of the entire tumor, including its deepest point and of the adjacent nonneoplastic mucosa on the same microscopic slides for reference.

(ii) *Shrinkage*. Most studies are based on microscopic measurement on glass slides which will very likely remain the gold standard. If a gross (prefixation/preprocessing) measurement is used, the value will likely be larger than the one on the microscopic slide due to tissue shrinkage during processing. Shrinkage is

likely to be more significant on the biopsy than in excision specimen. Reporting TT/DOI measurements on incisional biopsies is not recommended [141] since they may be unreliable [48].

(iii) *Tumor site*. As previously discussed, floor of mouth oral cancer was shown by several groups to metastasize at an earlier point than oral tongue [114, 117]; others reported no difference [142], and a recent study had opposite findings [118]. Further studies are needed to address the within and between subsite differences. Oral tongue various areas were reported to have different propensity to metastasis [1, 113]. While measurements are feasible and clinically significant in the floor of mouth, oral tongue, and buccal mucosa, they are of questionable value in subsites lacking submucosa such as hard palate or alveolar mucosa. Subsite variation does exist given that even in normal benign oral mucosa, thickness varies from 99 μ in the anterior FOM region to 294 μ in the such as buccal mucosa by optical coherence tomography. Thicker epithelia should be expected in the hyperplastic and dysplastic mucosa adjacent to the tumor compared to normal mucosa [119, 176]. Histoanatomic variance may exist between different areas within the same subsite such as different lymphatic vessel density and their relative position to the surface mucosa in the ventral compared to lateral oral tongue, which was proposed as an explanation [113] or between similar mucosal sites such as OT and FOM. Tumors with the same TT/DOI in FOM have higher potential to metastasize than buccal mucosa or tongue [113, 114, 117]. However, without using similar inclusion criteria and well-defined reference points, explaining the opposite results found by others [118] remains challenging.

(iv) *Measurement accuracy*. In many studies, DOI/TT is extracted from the pathology reports, likely generated by different pathologists with uneven expertise and probably using widely variable criteria [141, 167]. Most studies do not describe at all the way the measurement was obtained and what reference points were used. Lack of interobserver reproducibility studies or paucity of studies comparing the significance of DOI and TT is surprising given the plethora of papers on the topic of OSCC and tumor thickness (over 90 publications to date). As we have seen in our cohort [169], even when same deepest point of invasion is used, measurements made on the same slide using the same instrument can vary from one observer to the other.

Despite significant findings reported in a large multicenter study [142], the measurement may be difficult or unfeasible in large T3–T4 tumors since reconstruction needed would be difficult or impossible. Most studies focused on and the stronger significance is seen in early stage oral cancer (T1–T2). Some investigators even suggested restricting its usage for T1 oral cancer which seems entirely reasonable [179] although broad T2 tumors would likely benefit from the stratification.

Earlier requests to introduce this parameter in the staging system [161, 168, 177] were recently revived by a multicenter retrospective large cohort study leading to a new proposal for modifying current AJCC schema to include TT with cutoffs of

5 mm for T1 and 10 mm for T2–T4, respectively [142], currently awaiting further validation. Uniform or central measurements were not performed and methods of measurement and reference point(s) not specified in the study, and therefore inherent interobserver and intercenter variability was assumed by the investigators [142]. The new proposed system upstaged 61 % (279/454) of the current T1 tumors! In our hands, the new system upstaged 40% (21 of 52) T1 oral tumors (unpublished data). It is clear by the magnitude of this change that standardization of TT/DOI reporting is critical. Another recent proposal had a slightly different schema but similar cutoff including 5 mm thick tumors [160].

TT and DOI are incorrectly used interchangeably in the literature, and therefore data are often impossible to compare [141, 167]. This reinforces the importance of routinely, uniformly recording and correctly identifying TT and/or DOI. A consensus decision on which parameter (TT or DOI) should be used on the reference points, assessment of interobserver variability, and measurement standardization is imperative for clinical practice, tumor registry, and clinical protocol application. Two studies reporting both measurements in T1–T2 oral cancer identified a mean difference of 0.75–1.4 mm [166, 171]; one of them comparing TT and DOI in the same cohort concluded the latter was more predictive for nodal metastasis [171].

Clear correlation and prognostic value was demonstrated for T1/T2 tumors in multiple studies with a DOI of 4 mm being most predictive for oral tongue cancer [24, 141, 167]. Overall, the current data point to a DOI of 4 mm and/or TT of 5 mm to be used as cutoffs for elective neck dissection in oral cancer [137, 142, 160], with possible adjustments for floor of mouth tumors which, as discussed earlier, were shown to have a higher chance of metastases than oral tongue neoplasms at the same TT/DOI. However, one should be aware that well-conducted studies found cutoff values as low as 2 mm [180] to be significant when DOI measurements were imported the DOI value from pathology reports, subject to the variability described earlier. In fact, in later studies from the same group, a cutoff of 4 mm was significant [181], and this cutoff was confirmed by others in most recent studies [182, 183]. Rarely, OSCC may metastasize when DOI <2 mm as we have encountered in our practice and in a recent study of T1 oral cancer with rates of neck involvement of 7 %, 25 % and 39 % when DOI was <2 mm, between 2–5 mm, and >5 mm, respectively (unpublished data).

Given the shrinkage factor occurring after tissue fixation and processing, in vivo evaluation of thickness would be ideal. Measuring intraoperatively grossly the tumor thickness was not successful [171]; however, the possibility of intraoperative frozen section evaluation of thickness (routine practice in many centers for endometrial carcinoma, for instance) is an interesting proposition and may potentially be a helpful alternative in stratifying patients for elective neck dissection, avoiding unnecessary surgery in borderline cases, and helping optimal management [161, 171].

Alternative presurgical imaging measurements of oral mucosa or lesion thickness are promising either by ultrasound [184] or optical coherence tomography [119, 176, 185–187], but, in the absence of a strict pathologic definition of TT/DOI,

which currently remains the gold standard, they may be difficult to validate and apply in practice.

Currently the requirements that TT/DOI be reported in oral cancer dataset are not uniform. It is somewhat ironic and a reflection of the confusion and heterogeneity existing in the current literature that in the most recent CAP protocol for oral cavity cancer [11], recording the tumor thickness is considered facultative and several measurement options provided, while the most recent NCCN guidelines introduced the tumor "depth" >4 mm as a stratifying factor for treating the neck. In this algorithm, elective neck would be performed in tumors with DOI<2 mm only exceptionally, based on clinical judgment when DOI is 2–4 mm, and should be strongly considered when DOI>4 mm and no radiotherapy was planned [188].

One practical suggestion for clinical measurements and future research would be to reserve the term depth of invasion only for measurements to the base of adjacent nonneoplastic mucosa and use for the other reference points (i.e., adjacent mucosa, tumor surface) tumor thickness, specifying which method has been used. Importing thickness from the pathology report is suboptimal; in the single study where controlled measurements were compared with previously reported ones, DOI was more reliable than TT [169]. While some studies claim that a unique method was used by all pathologists in a given center, it is somewhat doubtful. Whenever possible, depth of invasion should be used [171].

Recording both tumor thickness and depth of invasion and the method used in a prospective fashion in the synoptic report will allow individual centers to adjust their practice. Variability in selecting and sampling the deepest aspect of tumor and differences in various measurements possible were recognized as critical factors as early as 1989 [104]. Unfortunately, very little progress has been made in obtaining interinstitutional standardization of these critical parameters. Both clinicians and pathologists should be aware of the difference between various subsites normal mucosa thickness and between TT and DOI since they may impact the clinical decision in T1 tumors where average DOI is 4.5 mm [169]. In summary, tumor thickness and depth are extremely important histologic parameters in assessing early oral cancer metastatic potential and strong independent survival predictors. Prospective, multicenter studies to assess the best, most feasible, reproducible, and predictive measurement method will be needed to validate the currently proposed cutoff measurement for tumor thickness of 5 mm. In the United Kingdom, tumor thickness is a required dataset element [12], whereas in CAP cancer synopsis it is optional [11].

5.2.4.4 Tumor grade

Oral squamous cell carcinoma (OSCC) has been routinely classified based on their grade of differentiation for almost a century. The original four-tier Broders' classification system [189] has historically morphed into a three-tier system with well-differentiated tumors resembling normal squamous epithelium to a large degree, poorly differentiated tumors exhibiting little or no histologic traits of the squamous phenotype and moderately differentiated tumors having an intermediate morphology between the two ends of the spectrum (Fig. 5.6).

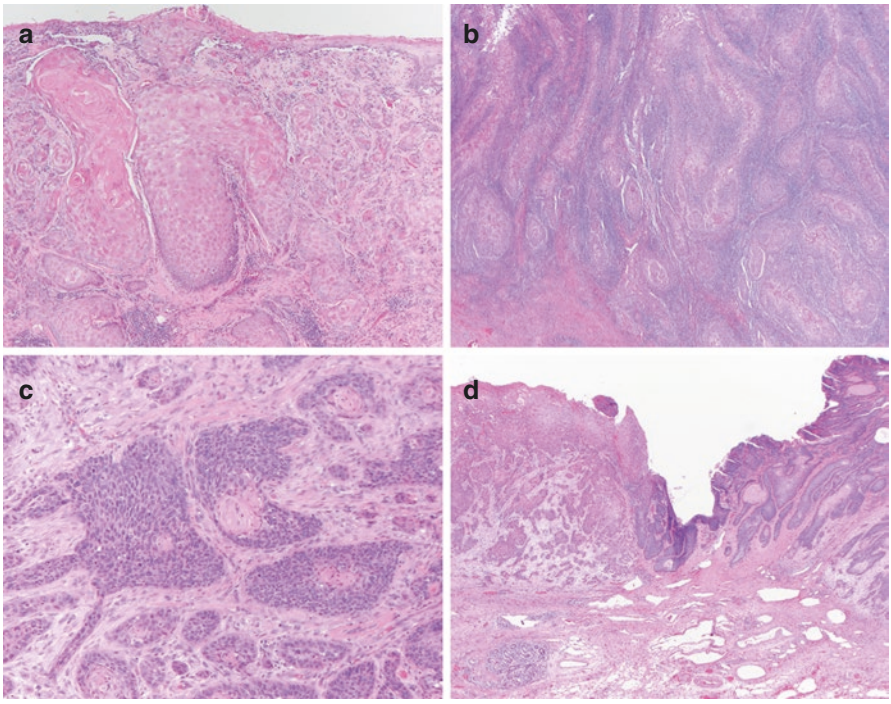

Fig. 5.6 Tumor differentiation. (**a**) Well-differentiated squamous cell carcinoma of the alveolar ridge had extensive medullary invasion and positive margins requiring a segmental mandibulectomy (not shown). (**b**) Moderately differentiated squamous cell carcinoma of the buccal mucosa with deep muscle invasion. (**c**) Poorly differentiated carcinoma of oral tongue. (**d**) Mixed patterns and variable differentiation can be seen in the same tumor; this floor of mouth carcinoma has two distinct components: one poorly differentiated, basaloid, nonkeratinizing (*right side*) with abrupt transition (*center*) to the moderately differentiated keratinizing component (*left side*)

Generally speaking, well-differentiated tumors have an indolent biology, whereas the poorly differentiated or undifferentiated tumors are aggressive and associated with negative prognosis in various oral subsites [44, 45, 65, 66, 94, 108, 113, 118, 135, 155, 156, 159, 190–194]. Many studies, however, found no significant correlation [46, 76, 111, 134, 195, 196], or the correlation was significant in univariate but not in multivariate analysis [120, 197, 198]. The tumor grade is distinct from the pattern of invasion although several investigators have used them interchangeably [112, 127, 132]. The distinction is important since often there is little or no correlation between tumor differentiation and pattern of invasion [134].

Several studies showed prognostic significance of tumor differentiation [199], but currently it is not considered an accurate prognostic indicator [11]. While poorly differentiated squamous cell carcinoma (PDSCC) is usually aggressive clinically, particularly in several oral cavity subsites (see earlier discussion), well-differentiated tumors can behave aggressively, infiltrating adjacent structures and

spreading to the lymph nodes (Figs. 5.10 and 5.11). Most oral SCCs are classified as moderately differentiated, further diluting this parameter's stratifying value [135, 165, 193].

Due to the subjectivity of the assessment, variable components in the same tumor, and interobserver variability [200], many deficiencies of tumor histologic grading likely have more to do with the tumor area studied and method of classification by individual observers. In general, the deeper aspect may be better predictor [18], and the highest grade should be documented in the pathology report. Alternatively, the prevalent grade may also be recorded [11] although we and others prefer recording the highest grade seen at the interface of the tumor-stroma, irrespective of component size [12].

Tumor differentiation is a reflection of neoplastic cells' retained capability of producing keratin, and distinction between keratinizing and nonkeratinizing oral cavity cancer is important. The vast majority of oral cavity tumors are keratinizing, and less than 5 % are poorly differentiated [121]. Keratinizing SCC is defined by the presence of extracellular keratin pearls, intracellular keratin, and/or intercellular bridges [1]. As stated, the vast majority of OSCCs are keratinizing, and exceptions should point toward rare variants (basaloid squamous cell carcinoma, below) or HPV-associated tumors, particularly in posterior oral subsites adjacent to the oropharynx.

Nonkeratinizing tumors are usually poorly differentiated carcinomas, or, rarely in oral cavity, undifferentiated. While all nonkeratinizing SCCs are by definition poorly differentiated, the reverse is not true: many poorly differentiated carcinomas show evidence of at least intracellular keratin formation. Mixed or even keratinizing phenotype can be seen in HPV-associated oropharyngeal neoplasms, and tumor localization is better than keratinization in stratifying the SCC needing HPV testing in oral cavity tumors.

In summary, tumor grading is generally considered to be an unreliable prognostic predictor. Predominance of moderately differentiated tumors in most studies, significant interobserver variability, and lack of defined uniform criteria to address tumor heterogeneity are all limiting factors. Grading or risk stratification systems based on or incorporating the pattern of invasion have been determined to be much stronger predictors of nodal metastasis and overall survival as will be discussed in the next section.

5.2.4.5 Pattern of invasion (POI)

POI at the tumor-host interface (tumor's deeper most aspect) has been shown to reflect tumor biologic potential for many decades. Generally speaking, as previously stated, exophytic tumors fare better than the endophytic, ulcerated, indurated, or deeply invasive ones [161, 171]. Not only depth but also pattern of involvement or tumor front shape has been shown to be extremely important: Tumors with pushing borders and a cohesive pattern of growth are associated with better prognosis than tumors showing small cell foci at some distance from mass bulk.

Several histologic risk score systems were proposed in the past to quantify these variable parameters including the pattern of invasion (POI) and tumor

differentiation, described originally in the larynx [131] and then adapted for oral cavity [111, 133–135] with various success rate in predicting outcome [121] defined as probability of neck metastasis, local and/or regional recurrence, disease-specific survival, and overall survival. Between the original system proposed in 1973 and subsequent unifying review and suggestion for a common strategy by Anneroth in 1987, at least five different modifications were suggested [201]. Two types of parameters were included in most systems: intrinsic tumor characteristics (keratinization, nuclear pleomorphism, mitoses, depth of invasion) and alterations at the tumor-stroma interface (mode/pattern of invasion, lymphoid response, desmoplastic reaction). Additional independent prognostic factors such as perineural invasion [122, 137, 202] or tumor size (T stage) [203], not included in previous iterations, were more recently proposed. Table 5.2 illustrates evolution of selected systems in the last decades, various parameters assessed and widely different cohorts studied.

These systems were dubbed "malignancy grading" [131, 201], "invasive front grading" [134], "histologic risk assessment" [122], or "clinicopathologic scoring system" [202]. Many of the histologic findings (nuclear pleomorphism, differentiation) are either subjectively assessed or can be widely variable in the same tumor. Unsurprisingly, most studies showed superior prognostic value of multifactorial systems when compared to tumor differentiation alone or to a single parameter but suffered from small sample and reproducibility issues [200].

Most recent systems were validated by the proposing authors [122, 137, 202, 203, 205], and to a degree predictive findings were confirmed by several groups in small cohorts of OSCC in different subsites [193, 600] in the oral tongue [177, 207, 208, 277] and lip [49], but not by others [139, 209]. This most recent scoring system was validated in a multicenter study with central review [122, 137, 205] identifying three risk groups (low, intermediate, and high) for locoregional recurrence and disease-specific survival. This is based on previous models but differs through a weighted score of various parameters and inclusion of perineural invasion, itself an independent prognostic factor. The pattern of invasion is essentially that of Anneroth for patterns 1–4 with the addition of pattern 5 for cases with tumor satellites away (>1 mm) from tumor perimeter [137, 205]. Depth of invasion is not part of this system. Several groups used this model, and one found it most predicting of OS when compared with other models [193]. Another group found that patient immunosuppression status played a more important role than histologic score [600]. It is difficult to compare these relatively small cohorts of different oral subsites. Reproducibility of histologic risk assessment model is also currently uncertain: a moderate agreement (kappa = 0.63) was reported in the single study to date [137]. As the author of the model noted, training of the pathologists is advisable before clinical implementation [205].

Related to the worse pattern of invasion is the concept of "tumor budding," imported in oral cavity cancer from other organs where it was proved to be associated with poor outcome. Tumor budding is defined by the presence of solitary cells

Table 5.2 Multiparameter histologic scoring systems for head and neck squamous cell carcinoma

	Histologic parameters evaluated												
System	Pattern or mode of invasion (points)	Structure (cell cohesion)	Nuclear pleomorphism	Differentiation/ Keratinization score (points)	Mitotic activity	PNI (points)	LVI	Lymphoid response	Tumor thickness or stage of invasion[a]	Tumor stage	Tumor site, cohort, specimen, outcome	Histologic grade score range	Risk or outcome stratification (points)
Jakobsson [131]	1–4	1–4 (papillary-1; single cells-4)	1–4	1–4	1–4	NA	1–4	1–4	1–4[a]	NA	Larynx, 42, biopsy, survival	8–32	Better (≤20) Worse (>20)
Anneroth [201]	1–4	NA	1–4	1–4	1–41	NA	NA	1–4	1–4[a]	NA	–	6–24	–
Bryne [134]	1–4	NA	1–4	1–4	1–4	NA	NA	1–4	NA	NA	Buccoalveolar, 68, biopsy, survival	5–20	Better (5–10) Worse (>10)
Bryne [111]	1–4	NA	1–4	1–4	NA	NA	NA	1–4	NA	NA	Floor of mouth, 61, biopsy, survival	4–16	Good (4–8) Moderate (9–12) Poor (13–16)
Martinez-Gimeno [202][b]	Uniform/ others (1/3)	NA	NA	1–3 (1/3/5)	NA	No/yes (1/2)	1/7	High/low (1/3)	1/3/5 (<3/3-7/>7 mm)	T1–4 (1/3/3/5)	Oral cavity, 80, resection, neck involvement	7–30	I (7–12) II (13–16) III (17-20) IV (21–30)
Brandwein-Gensler [122][b]	1–5 (0/0/0/1/3)	NA	NA	NA	NA	No/ small/ large nerve (0/1/3)	NA	1–3	NA	NA	Oral cavity, 292, resection, overall survival and locoregional recurrence	0–9	Low (0) Intermediate (1–2) High (3–9)

PNI perineural invasion, *LVI* lymphovascular invasion, *NA* not assessed

[a]Noninvasive (in situ) carcinoma included, stage based on the histoanatomical structures involved

[b]Weighted point system

or clusters of less than five malignant cells at the interface between tumor and stroma (Fig. 5.7). This finding has recently shown to harbinger occult neck metastasis by several groups in early-stage oral cancer [139, 140, 210–212].

Most previous studies demonstrated strong correlation between the pattern of invasion (regardless of scoring system used) and neck metastasis [113, 118, 135, 204] and survival even when the histologic score system employed was not statistically significant [139]. Table 5.2 illustrates the broad differences between various systems. It is clear that certain parameters have an independent predictive value (perineural invasion, pattern of invasion, lymphovascular invasion) and they could be used either isolated or part of the comprehensive system in an attempt to quantify the intrinsic tumor aggressiveness in general and the likelihood of locoregional recurrence in particular. The interrelationship between different histologic parameters should be solved through multivariate analysis and regression analysis in a sufficiently powered study, preferably from the same subsite and with central pathology

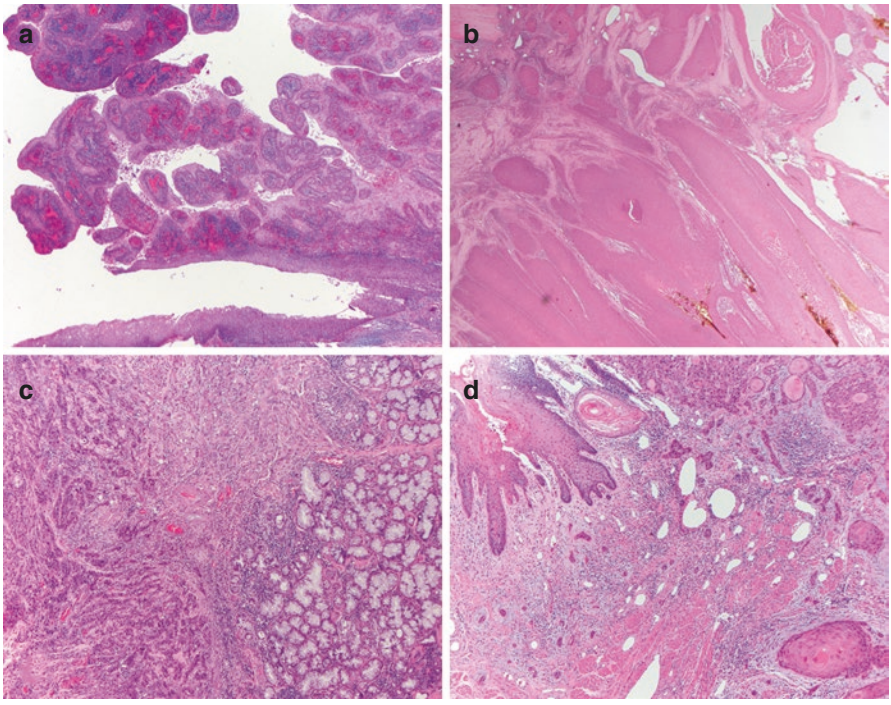

Fig. 5.7 Patterns of invasion. (**a**) An exophytic pattern of growth as seen in this buccogingival tumor is associated with good prognosis. (**b**) Verrucous carcinoma can be locally destructive, despite its bland morphology, in this gingival tumor involving the mandibular bone (*left upper*), but does not metastasize. (**c**) This gingival tumor shows an infiltrating pattern with small tumor cell clusters involving minor salivary gland. (**d**) Aggressive pattern of invasion is seen in this 19-year-old man's oral tongue carcinoma with small tumor cell clusters (*left lower*) infiltrating muscle away from the tumor bulk (*right upper*). Tumor budding is extensive here, defined as less than five cells present in a cluster

review [208]. We have seen an association between PNI and POI in T1 oral cancer (unpublished data), likely a reflection of intrinsic neoplastic aggressiveness.

Albeit not universally accepted and currently lacking consensus on which system one should use, recording the POI either as a score or more general/descriptive fashion is recommended since it may provide prognostic information and guide management in selected patients until a scoring system will be prospectively validated and agreed on for routine clinical use. In a currently ongoing study, we found that even in early, superficial tumors and when using a binary stratification into nonaggressive (1–3) and aggressive (4–5) POI, a significant number of cases required consensus review and extensive discussions. Tumor budding may be also recorded since in our experience reflects an aggressive POI (patterns of 4 or 5) as reported by others [139].

In summary, POI is a strong predictor of locoregional recurrence and overall survival; however, due to uncertainty on best scoring system and until multicenter prospective validation, it is currently not a required element in the College of American Pathologists guidelines [11], whereas RCP guidelines require recording the pattern of invasion using a binary system (cohesive cell groups or more than 15 cells/groups vs. non-cohesive or individual or small cell groups [12]).

5.2.4.6 Perineural invasion

PNI presence is a harbinger of aggressive disease and an independent predicting factor of local recurrence and decreased survival, confirmed in multiple studies in head and neck cancer in various locations [46, 56, 104, 113, 118, 145, 208, 213–216]. In some studies, it was the only or most important predictor of neck metastasis or survival [144].

Lack of a consensus definition of PNI and lack of clarity in describing inclusion criteria for PNI in multiple studies are at least in part responsible for the widely variable reported incidence of perineural invasion in oral cancer ranging from 14 to 63 % [147]. A recent proposal suggested that at least one-third of the circumference perineurium should be involved or any cells identified within any of the three nerve sheath layers (epi-, peri-, and endoneurium) as minimal criteria for neurotropism [217].

Perineural detection in routine clinical practice is likely underreported, and its rate changes at re-review depending on the criteria used and ancillary studies employed [148, 218, 219]. In two studies, the number of PNI-positive cases approximately doubled at targeted re-review of hematoxylin-eosin-stained slides and almost tripled after using immunohistochemistry (S100), which increased the positive rate from 30 % to 82 % and from 22 % to 51 %, respectively [218, 219]. These high rates raise the question of accuracy of S100 usage which may overestimate PNI's presence without a rigorous definition. In almost all cases in clinical practice and most previously published studies, these ancillary studies are not routinely performed, and subtle involvement is probably overlooked in a percentage of cases, depending on the extent of this finding, sampling, and pathologist's determination. Considering that the above results came from large referral and academic centers, it is easy to understand the variability of results in literature.

PNI detection accuracy is highly dependent on the pathologist experience and tenacity, sampling, and criteria used. A careful histologic examination is warranted to avoid underdetection of small diameter nerve involvement; however, overdiagnosis may be equally problematic given its clinical implications. In a recent study, using only morphologic assessment, reclassification of perineural invasion status was reported in 19 % (27 of 142) of head and neck squamous cell cancers studied; of note, most of these were originally misclassified as positive (21 of 27) in the pathology report, and only 6 of 27 were false negative at re-review [148]. The tumor cells diffusely involving soft tissue, including nerve, should not be labeled as perineural invasion (Fig. 5.8). As earlier stated, perineural invasion may contribute to bony involvement through alveolar nerve and alveolar canal spread [75].

Several studies show that invasion of large nerves is more significant than small nerves (less than 1 mm diameter) [122, 219], but other studies could not confirm this separation [220]. Others could not find any large nerve involvement in a recent large

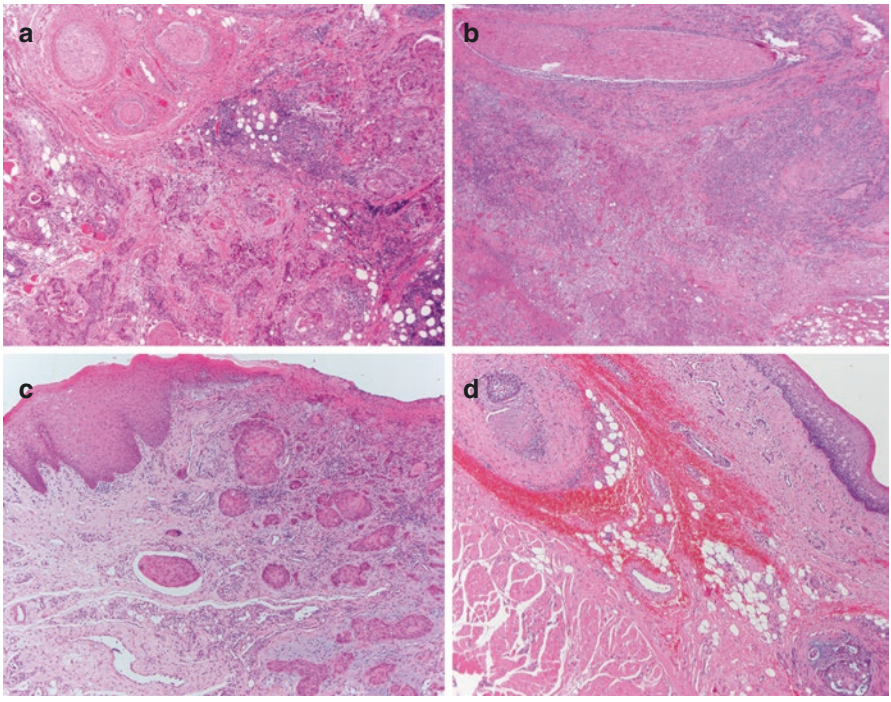

Fig. 5.8 (**a**) Perineural invasion (PNI) is seen in several small neural fibers (*left upper*) away from the main tumor (*right* and *lower*) and is associated with worse prognosis. (**b**) Recurrent tumors often present with extensive perineural involvement as seen in this buccal cancer, circumferentially involving large nerve (*upper*) and many smaller neural fibers (*right*). (**c**) This well-differentiated squamous cell carcinoma of the oral tongue is present in the lymphatic space. Ipsilateral neck lymph nodes were negative, but tumor recurred in the contralateral neck 6 months after surgery. (**d**) Tumor involves the submucosal vessels in this oral tongue carcinoma and spread to submental lymph nodes

series [221] which is similar to our experience in early oral cancer. It seems more likely that the PNI's extent and its localization in relation to the tumor front are more important, likely gauging intrinsic tumor neurotropism. Recent studies show that when the involved nerve is located outside the tumor (extratumoral), it may be more significant than when it is found inside or adjacent to the tumor (<0.2 mm from invasive front) [148].

Increased local recurrence of tumors with PNI is classically considered to be related to "skip" progression throughout the perineurium although several studies could not prove this putative mechanism. Mucosal and cutaneous SCCs have inherent biologic differences and should be discussed separately including their neurotropism [601]. Further studies for site-specific differences are needed to definitively answer this question in oral cavity, but most studies confirm that PNI is an independent risk factor for decreased overall, disease-specific survival as well as increased local and locoregional recurrence. PNI increases the risk of nodal disease and rate of occult metastasis [56, 113, 118, 143, 146, 172, 202, 220, 222, 356]. A recent large study from Taiwan on early oral carcinoma suggested elective neck dissection when PNI was present [56] and observation when PNI and LVI were both absent [224].

Different results may be related to variance in definition, detection rate, number of cases, and type of patients included (in post-adjuvant radiotherapy, recurrent OSCC PNI is extremely common) [233]. Many studies import this information from the pathology reports rendering therefore questionable results given uncertain accuracy of PNI clinical detection in routine practice. Even in academic centers, this parameter was reported as missing from reports in up to 41 % of cases in a recent study [143].

Perineural invasion (not otherwise specified) is regarded as an adverse indicator in current clinical management algorithms (NCCN), and its presence may indicate a need for adjuvant therapy. PNI documentation in the pathology report is required [11, 12], and diameter of the largest involved nerve and its localization (intra- or extratumoral) could be included in positive cases for local validation of this finding. Both false-positive and false-negative results are detected frequently at retrospective review.

5.2.4.7 Lymphovascular Invasion

The presence of lymphovascular invasion (LVI) is concerning for, but does not always accurately predict, an increased risk for nodal metastasis. This finding is currently considered to be a weak predictor of nodal disease [12] although several independent groups have recently confirmed its poor prognosis in oral cancer [56, 113, 166, 182, 225].

The presence of LVI is associated with nodal spread or occult metastases [113, 145, 146, 172, 183, 226]; local and locoregional recurrence [225, 227] or a statistical association with nodal metastasis was reported in univariate but not multivariate analysis [118, 145, 166, 222, 228, 229]. Others, however, did not find a positive correlation between LVI and outcome [135, 216, 220, 230, 231]. A recent large study from Taiwan combined PNI and LVI and reported a higher negative predictive value for neck involvement of early oral cancer without PNI and LVI than tumor stage and

thickness [224]. Similarly to and probably even more so than in perineural invasion, this finding is likely underrecognized and underreported by pathologists in routine clinical practice. Its detection increases slightly at focused re-review and more significantly (20%) when immunohistochemistry is used [218]. Nicely illustrating the suboptimal detection in the specimen or underrepresentation in pathology reports, a recent retrospective study of 533 patients with oral cancer reported that while 53% of patients had nodal involvement, LVI was present in only 2% [217]. This is the reason why most multifactorial histologic scoring systems do not include lymphovascular invasion with rare exceptions [131, 202]. However, when the presence of LVI is recorded through a focused pathology review, lymphovascular invasion was identified in 10 of 78 (13%) cases and had 100% positive predictive value in detecting occult metastasis in sentinel lymph node biopsy performed for early oral cancer [166].

The main pitfalls are retraction artifact and tumor cells "carryover" (dislodged) into vascular spaces. Diagnosis implies subjective evaluation and requires some experience. When easily identified in multiple lymphatics (so-called lymphangitic spread) (Fig. 5.8), that particular tumor is very likely to metastasize, but a formal quantification system does not exist. Similar to PNI, LVI is considered a high-risk histologic feature in clinical management [188, 232, 602] but fraught by similar issues of nonuniform definition, lack of quantification, and possible underdetection of LVI in routine practice. In the hands of experienced pathologists, however, LVI detection was associated with tumor site, tumor thickness, pattern of invasion and histologic grade, positive resection margins, local and locoregional recurrence, and survival [234].

Unfortunately, LVI status is missing from 30% of the pathology reports [234] although it is a mandatory data point to be recorded in the synoptic report but separation between lymphatic and venous involvement is not required since histologically it may not be reliable [11, 12].

5.2.4.8 Bone invasion

Bone invasion is a harbinger of aggressive biology in head and neck cancer and is incorporated in the current staging system for OSCC [15] and occurs in approximately one-fifth of oral squamous cell carcinomas [235]. Mandibular bone is by far the most commonly involved in OSCC (>90%) by tumors of gingiva/alveolar mucosa, retromolar trigone, floor of mouth, and tongue.

Various patterns of bone involvement exist and have been described by several groups: through the mandibular periosteum, with an erosive or infiltrative pattern [69, 74, 76, 78], but the most common point of entry appears to be the junction of reflected and attached gingival mucosa [71, 74] in both dentate and edentulous patients. These mechanisms and histology were discussed in more detail under gingival cancers.

Deep (i.e., medullary) bone invasion has been associated with increased local recurrence and decreased survival [236], although recent studies suggest its impact on OS may not be that strong [235, 237].

The distinction between cortical *erosion* and medullary *invasion* is essential since the former do not qualify for T4 designation [11, 12, 15, 238]. The designation of cortical invasion criteria in several clinical papers is not always clear, and

although most of these large studies are based on the histopathologic evidence, this is imported from pathology reports [235, 237, 239], and lacking uniform pathology review to confirm or refine the original definition and assure adequate sampling. Pathologic classification in one of the three subgroups (negative, superficial and deep bone invasion) is expected to vary, possibly quite widely, based on individual tumor sampling and pathologist experience with this assessment.

Bone processing may present challenges at the bench, and decalcification may be performed after sampling the margins and other key mucosal and soft tissue sections, since sampling the soft tissue attached to the bone is important to predict recurrence [67].

Histologically, this distinction may not be straightforward in two-dimensional image provided by histologic slides, and several levels may be needed to clarify minimal medullary involvement. Unsuspected bone invasion is common in oral tumors despite current sophisticated imaging [240] in tumors close (within 1 cm) from the mandible [241], and imaging appearance does not always correlate with histologic pattern of involvement [242]. Tumors with deep (medullary) bone invasion and a high POI are worse [67, 191]. Negative bone margins are essential [240, 241], but

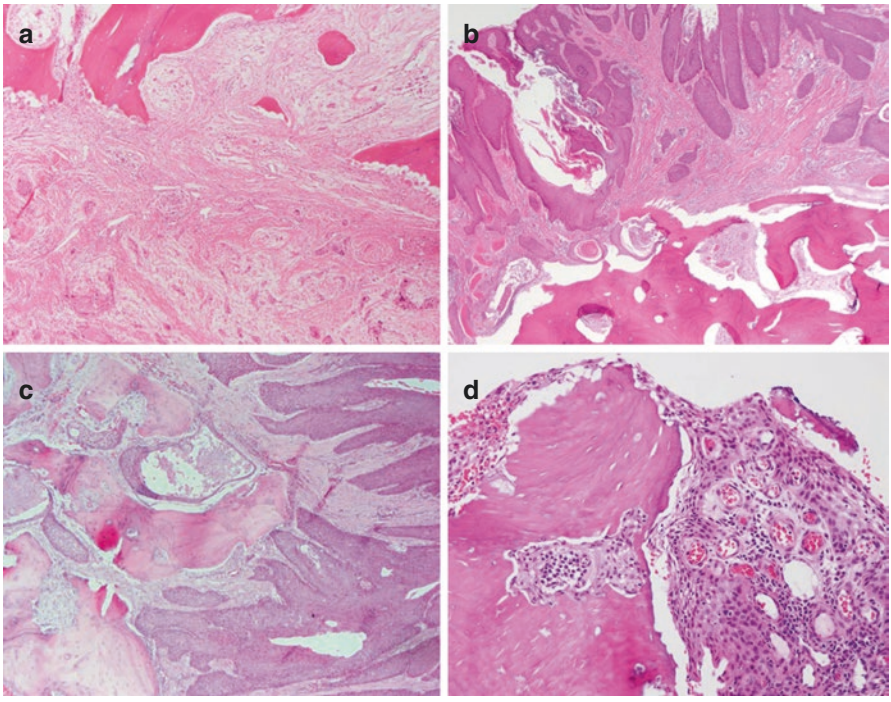

Fig. 5.9 (**a**) Erosion with osteoclastic reaction (*upper*) without medullary involvement was seen in this retromolar trigone carcinoma (*lower*). (**b**, **c**) In contrast, in this alveolar ridge squamous cell carcinoma, tumor cells penetrate into bone marrow space. Note desmoplastic reaction surrounding the tumor. (**d**) Bone pseudoinvasion is often seen associated with jaw osteonecrosis. Desmoplastic reaction is absent, and epithelial cells with mild atypia are juxtaposed to necrotic trabecula

others showed that adjacent *soft tissue* margins may drive recurrence, particularly in tumors with an infiltrating pattern, extending deeper than 10 mm into soft tissue [67].

In clinical practice, bone margins are not routinely evaluated intraoperatively due to feasibility and need for decalcification. In the rare reported attempts, frozen section of margin medullary curetting [244, 246], drill-obtained sample [245], or cytologic evaluation of medullary scraping [247, 248] yielded various results with sensitivity ranging from 50 to 89 % [244].

In the previously irradiated bone, the involvement can be extensive since necrosis and secondary changes allow tumor penetration [239], and a segmental mandibulectomy is the treatment of choice in these patients [78].

Although histopathologic evaluation remains the gold standard of establishing bone invasion, there is a need for uniform sampling guidelines both qualitatively and quantitatively of the bone adjacent to the primary tumor.

Many clinical papers [235, 237, 239] discussing the difference between cortical erosion and medullary invasion offer limited or no information on the type and thoroughness of sampling (or even if the bone was evaluated microscopically!), and many lack pathology re-review for uniform classification [237, 240] or do not separate between cortical and medullary involvement [240]. Virtually all studies to date fail to report the number of sections evaluated or strategy of sampling. Studies with thorough pathologic evaluation correlated with outcome are the exception [67].

Cortical erosion should not be classified as T4 [11, 15, 235, 237, 238]; this upstaging of small oral tumors has been recently questioned even for superficial cortical bone invasion [235, 237]. Bone invasion presence and depth (cortical versus medullary) are considered mandatory data for the pathology synoptic report [11, 12].

5.2.4.9 Surgical Margins in Oral Cancer

Complete tumor excision is the single most important factor determining patient cure in HNSCC in general and OSCC in particular. Presence of tumor at or close to the resection margin is associated with locoregional recurrence [99, 249, 250] and decreased overall or disease-specific survival [87, 106, 194, 229, 249–251]. The final status of surgical margins will predict the risk for local and regional recurrence and overall survival and dictate necessity of adjuvant therapy.

Assessment of the resection margins is performed by the pathologist in close collaboration with the surgeon. While the ultimate responsibility for margin designation status lies with the pathologist, reaching an *accurate* conclusion is determined by several factors including adequate communication with the surgeon; standardized, uniform, and targeted margin sampling strategy; locally developed well-defined and consistently applied protocols, flexible enough to adjust for various oral subsites; and specific tumor localization. Other factors that would influence the likelihood of an involved margin such as pattern of infiltration, perineural involvement, and microscopic depth of invasion are unfortunately mostly unknown at the time of surgery. Evaluation of these additional factors could be performed intraoperatively in a well-selected deep section of the tumor, and findings may be applied in extending the revision of the surgical margins as indicated and feasible on each case.

Surgical anatomy of the oral cancer specimens can be extremely complex, and correct orientation is critical. Surgeons should realize that whenever a specimen is received in the pathology department without proper orientation, the adequate margin assessment is already compromised. Tissues change *ex vivo* even before fixation, and not uncommonly surgeons asked to orient a specimen after being retrieved from formalin or even postoperatively will find the task as challenging as the pathologist. Precise orientation is a prerequisite but only the beginning for a meaningful surgical margin evaluation. Ideally, both the surgeon and the pathologist should jointly analyze the specimen and identify the critical areas and closest margins as determined by the preoperative imaging, intraoperative surgical findings, and pathologic examination of the specimen to determine the closest tumor margins(s). If this is not feasible, complete careful labeling of all margins and identification of clinically closest margins are essential for best pathologic sampling and obtaining an accurate and predictive measurement of the clearance.

There are two distinct prevalent sampling strategies at the surgical margins: the so-called specimen driven (en bloc), when the margins are assessed by the pathologist in relation to the entire specimen, and defect driven, in which the surgeon submitted the tissue sampled from the "tumor bed." The former was endorsed in a recent review of International Head and Neck Scientific Group [150] and in the pathologic guidelines [11, 12]. However, at least in the United States, over 90 % of the surveyed head and neck surgeons favored the tumor bed sampling [252]. In reality, the vast majority of surgical specimen margin in the head and neck cancer are submitted separately from the tumor bed [252], approach found to be inferior by several groups and associated with local recurrence even when revision margins were negative [129, 152, 221, 253]. Local resources and other logistical obstacles may preclude the optimal approach, but a joint effort from the pathologists and surgeons is desired in referral and academic centers, if not throughout all systems, to redress the clinical practice of margin assessment.

Widely variable definitions of what constitutes an adequate margin exist in the literature and among various institutions or even among different surgeons at the same hospital. Generally, 5 mm is deemed an overall minimum clearance to be accomplished in OSCC [150]. While transected margins are universally accepted as positive, definition of a close and negative margin widely varies among surgeons [252]. A "close margin" was defined as within 2 mm [254] and 3 mm [255], but most of literature, current guidelines, and some large randomized trials used 5 mm as the arbitrary cutoff [150, 188, 252]. Currently accepted definitions of positive, close, and negative margins in oral cancer are cut through (or less than 1 mm), 5 mm or less, and >5 mm, respectively [11, 150].

The pathologist should report all resection margins to include *mucosal*, *soft tissue*, *and bone* margins in general practice of OSCC [11, 12, 150]. A resection margin is defined as any tissue plane where "the surgeon knife" met the tissue, and distance to the closest inked (or cauterized) margin should be measured by the pathologist microscopically from a perpendicular section to the plane of resection [11, 12, 150, 188].

Dysplasia or carcinoma in situ present at the margin is another currently unresolved topic. The same survey showed that most surgeons consider positive only the margins with carcinoma in situ but not dysplasia, and in this author experience, this is not limited to private practice but also encountered in the philosophy of few surgeons in academic and tertiary care centers. Similarly, rare studies included dysplasia/carcinoma in situ in their definition of positive/close margins [99], whereas the vast majority do not [252, 255]. Of note, the prevalent opinion in the pathology literature is that high-risk dysplasia should be reported intraoperatively [11] and clinically addressed when feasible [188] – an opinion that we share – and this finding is communicated to our surgeons during intraoperative consultations. Based on surgeon preference and anatomic feasibility, revision of margins is performed in most if not all high-risk dysplasia cases. Patients with extensive high-grade dysplasia or carcinoma in situ can be treated more conservatively. To complicate matters even further, moderate dysplasia has been traditionally classified into either high-grade or low-grade dysplasia category by various authors – as it will be discussed in the next section. High recurrence rate was reported in the margins with *any* degree of dysplasia in a small cohort [109].

Surgical and Pathologic Sampling of Resection Margins. Samples can be taken by the surgeon from the surgical bed or by the pathologist from the en bloc specimen. As discussed, many pathologists [11] and some surgeons consider that the latter is superior [150] as recently compelling demonstrated in two multicenter retrospective studies of oral tongue cancer with central pathologic review [152, 221].

Soft tissue (deep aspect) may be more commonly involved than mucosal margins [256–258] although the latter comprise the vast majority of samples submitted by head and neck surgeons for frozen sections. The amount of submucosal tissue that is sent to pathologist for frozen section is limited, usually one section of the "deep" margin (personal observation). In a histopathologic study of involved margins in oral cancer, 87 % of all positive margins included deep soft tissue margins and only 16 % involved the mucosal margins [257].

Bone margins have been previously discussed. Several methodologies have reasonably good results including cytologic scraping or curetting of the bone marrow and cancellous bone at the resection margins intraoperatively. No guidelines exist regarding horizontal margin in marginal mandibulectomy. Our practice is to block out the segment of bone closest to the tumor and submit it entirely for microscopic examination.

Frozen section diagnoses are usually accurate [259–262], and they help in achieving local control [263]. Pathologists should sample more extensively selected frozen section margins to avoid false-negative results [263, 264]. Discrepancies between frozen and permanent sections occur in less than 5 % of margins performed for oral cancer [259].

Before surgery, several factors should be considered including tumor size and site [266]. Large size tumors are more likely to be associated with positive margins: In one study conducted in a center with uniform, standardized pathology evaluation, T1 oral cancers had 7 % involved margins, whereas 39 % of T4 tumors

had positive margins [257]. Maxillary alveolar, posterior (retromolar), and buccal tumors (in descending order of frequency) had higher rates of positive margins than floor of mouth and tongue [257]. Similar findings were reported in a recent National Cancer Database review with 7 % positive margins in low stage oral cancer. The positive rate was higher in large tumors, certain subsites (floor of mouth, buccal, retromolar trigon), intermediate- to high-grade histology, and small volume centers [108].

Attempts to correlate the clinical preoperative estimate of tumor extension and final histopathologic margins showed also subsite and stage discrepancies of 59 % (range 42–72 %) [89]. Tissue shrinkage immediately after surgical removal and prior to histologic processing definitely plays a role but provides only part of the explanation [267]. It is perhaps relevant that in most studies pathology margin status was extracted from the pathology reports. This wide range illustrates both the difficulty in adequately assessing the preoperative size and controlling for confounding factors such as tissue shrinkage. PNI was associated with positive margins [129] and carries an intrinsic independent risk for local recurrence (see above) particularly when associated with large neural fibers or present outside the tumor contour.

Several *practical considerations* in margin sampling and evaluation which may influence their accuracy and predictive value are listed below: (i) surgical sampling, as already discussed, is most commonly by intraoperative examination of separate biopsies from tumor bed and only rarely though en bloc exam; (ii) adequate sampling of the submucosal soft tissue is essential both by the surgeon or at the time of gross examination at the pathology bench; (iii) *en face* versus perpendicular sampling: in our laboratory, we discourage all prosectors to obtain parallel or *en face* sectioning of the margin which do not allow a reliable measurement and carry the risk of false-positive results in thick, uneven sections; and (iv) tissue shrinkage has been considered traditionally to play a major (negative) role in the accurate reporting of oral cancer margins. The estimated 46 % reduction due to combined postsurgical ex vivo retraction and fixation-/processing-induced shrinkage [266], estimate derived from animal studies [268], is unlikely with current processors [150], but at least 22 % tissue contraction should be expected in oral tongue and floor of mouth [89, 267]. Differences are site and stage dependent and may be 10 % or even less in larger tumors [150, 267]. Accurately measuring the presurgical clearance is problematic; as any pathologist experienced in gross evaluation could attest, the tumor contours could be elusive even when the entire specimen is serially sectioned and assessed at the grossing bench immediately postoperatively and are better delineated after fixation. Studies reporting clinical uniform presurgical radial margin measurements of 1 cm appear more geometrical than majority of specimens encountered in this author's experience [269].

Not only surgeons variably sample the margins but pathologists reporting of the clearance was reported missing in over one-fourth of the cases in a cancer center [234], together with other important histologic prognosticators. Faulty communication and at times disconnect between surgeons and pathologists on the relevant margins, localization and significance of frozen sections margins biopsies, and lack of a common strategy transpired from the two large surveys of the two groups [252, 270].

An improvement of frozen section results and clinical outcome using a team approach and well-defined protocol was recently reported in a single center experience [271].

Other frozen section challenging morphologic findings that may impact its accuracy include thermal artifact (which may obscure small foci of tumor), high-grade dysplasia or carcinoma in situ extending into the salivary ducts and mimicking invasive cancer, pseudoepitheliomatous hyperplasia, dense lymphoid response obscuring the tumor, extensive giant cell reaction, tonsillar mucosa, or lymphoepithelial cysts – most of which will be discussed in the differential diagnosis section below.

Conceptual Considerations. Not all recurrent tumors are residual or persistent; in broad lesions with extensive intraepithelial neoplasia, a new primary lesion is a distinct possibility in the field effect. Most clinical studies import the pathologic clearance from the reports and therefore are subject to multi-observer variability [99, 106, 255].

A 2–4 mm fragment of tissue is unlikely to be representative for a 2–4 cm mucosal margin, and pathologists have expressed discomfort in reconciling positive margins in the main specimen with negative findings in the intraoperative consultation [270]. It has been shown that in patients with initial frozen section positive margin, the recurrence rate was higher even if negative margins were eventually attained after revision [129, 221].

In tumors with submucosal extensive spread or high-risk infiltrative pattern of growth, deep submucosal tissue should be sampled extensively by the surgeon or pathologist intraoperatively. Unfortunately, the pattern of invasion is usually best determined after evaluating the entire tumor in the excisional specimens and usually is not known at the time of surgery [122].

Subsite differences exist based on local histoanatomy. While in laryngeal cancer where margins of 1–2 mm can be adequate, different intraoral cancers have variable risk for local recurrence.

The distinction between oral carcinoma in situ (in fact, a rare occurrence in oral cavity if strictly defined) and moderate/severe dysplasia is often irrelevant for many pathologists [1, 11, 12], whereas many surgeons would address intraoperatively only the former but not the latter if present at the margin [252]. Surgeons need to be aware of the lack of reproducible difference within the high-risk lesions, and pathologists should probably abandon the four-tier system and use instead a two-tier one, at least for the frozen section margin reporting, helping the surgeon in stratifying the areas needing additional management.

There is clinically no consensus on postsurgical management of close and positive margins, but some form of adjuvant therapy, frequently radiotherapy, is usually employed [150] although this indication has been recently contested [272]. Certainly the margin definition, patient selection, and employed methodologies play a significant role in single center results variability. In two recent prospective randomized trials assessing the value of chemotherapy in positive margin, definition of positive margins was incongruent (“at the margin” vs. <5 mm) [150]. Currently undergoing studies with a centralized pathology review and measurement, site and stage restricted, may help clarify residual questions [152]. Similar prospective multicenter studies

sufficiently powered would be ideal to guide clinical practice but are logistically challenging [275].

In sum, adequate surgical margin clearance is essential for local control. The definition of an adequate margin in oral cancer is not uniform but 5 mm is the most accepted cutoff. Surgical margins reporting and their clearance (in mm) is a required pathologic data point in the oral cancer synoptic reports [11, 12]. There is currently wide variability in its definition, surgical sampling, and pathologic assessment in different institutions and studies. A uniform consensus approach [150] will allow evidence-based comparison of various centers' experience and improve outcome. Submucosal and soft tissue margins should be sampled adequately, the clearance measured and reported for both the invasive tumor and high-grade dysplasia. Specimen-driven margin evaluation is optimal and should be employed where feasible. Close communication between the surgeon and pathologist is of essence for the correct assessment, measurements, and clinical decision.

5.2.4.10 Tumor lymphoid response

TLR at the tumor front interface with normal tissue is considered a surrogate marker for antitumor immune response, and several studies reported a survival benefit associated with increased density of lymphocytes. These studies showed that increased lymphoid response at the tumor-host interface correlated with better prognosis in oral cancer [138, 209], that decreased TLR correlates with nodal metastasis [49, 190, 208] and poor survival [122, 274] and that TLR predicted response to radiation [209]. Others, however, found no significant association with outcome [139].

TLR has been included in most histologic multifactorial scoring systems since the original ones to the most recently proposed [122, 131, 134, 203]. It may be more prominent in certain subsites as tongue than other locations [209] and comprises predominantly cytotoxic T cell [275]. TLR may harbinger early stromal invasion in incipient carcinoma (Figs. 5.1, 5.2 and 5.14). It should be differentiated from other inflammatory responses to various keratin related antigens such as peri- or intratumoral neutrophilic or eosinophilic infiltrate and foreign body giant cell reaction (Fig. 5.14).

While emerging evidence that local lymphoid response plays a significant role in tumor biology and even outcome, this parameter is currently considered nonessential for oral cancer synoptic reports [12]. Further validation of its significance and scoring standardization is desirable before its consideration for clinical practice application.

5.2.4.11 Stromal Desmoplastic Response (SDR)

Invasive oral cancer is not uncommonly associated with desmoplasia, a histomorphologic term denoting mesenchymal reaction immediately adjacent to invasive foci, usually associated with a myofibroblast proliferation at the tumor-host interface. Stromal reaction/desmoplasia was found to identify an aggressive phenotype in a cohort of patients with cutaneous SCC including the vermilion [278].

Desmoplasia is less studied in oral mucosal carcinoma; however, increased (myo) fibroblasts at tumor-stroma interface were shown to negatively impact prognosis in oral cancer by several groups [207, 277–279]. In some studies, SDR was the predicting factor for mortality in oral tongue squamous cancer [280], but the same group

could not reproduce this finding in a larger subsequent cohort [139]. Currently there is no uniformly accepted system of evaluating desmoplastic response or its relative composition of the fibroblasts and myofibroblasts. Definitions that require immunohistochemical studies [280] significantly limit clinical applicability of these findings.

For clinical practice reporting, SDR is considered a nonessential dataset point in some guidelines [12] or not addressed in others [11].

5.2.4.12 Histologic Prognosticators: Summary

In summary, various clinical and histologic parameters should be documented in the pathology report. While interrelation between various histologic features may reflect intrinsic tumor aggressiveness, a combined score may be helpful. Difficulty resides in separating the powerful predictors (perineural invasion) from the weaker ones (tumor grade), assigning relative significance, and obtaining a uniform assessment and reporting for each. Lack of uniformity, imprecise definition (tumor thickness/depth of invasion), various oral subsites reported together in the literature are major obstacles in updating current staging schema and therapeutic algorithms. Histologic data imported from databases are probably suboptimal given the definition variance and interobserver variability (thickness, margins), and multiple models used (pattern of invasion). A uniform, centralized, or consensus pathology review would be ideal for future studies. The final aim for each patient is to incorporate all relevant clinical characteristics preoperatively and complete them with the tumor histologic score and other parameters discussed above in order to obtain a modular prognostic system, which will drive the individual management [281].

5.2.5 Squamous Cell Carcinoma Variants

As previously stated, most of the discussion above pertains to the conventional keratinizing squamous cell carcinoma, which comprises the vast majority of oral cancer. However, OSCC is a heterogeneous tumor, with variable keratinization, pattern of growth, and architecture. Not uncommonly exophytic-papillary projections are seen in predominantly endophytic tumors, and such tumors should not be classified as papillary variant. Some variants are important to recognize for their indolent, nonmetastatic (pure verrucous, papillary), or aggressive (basaloid, sarcomatoid) biology, but other alterations (acantholytic, adenoid-like changes) are so common that it is doubtful that they are bona fide variants carrying a specific biologic potential and not simply histologic patterns. Several WHO-recognized subtypes of oral cancer are discussed here and their potential clinical implications are reviewed. Some of these variants are better described than others, but with the exception of verrucous carcinoma, most are quite rare in the oral cavity and much frequent in other areas of the upper aerodigestive system or skin.

One challenge in precisely identifying these variants of squamous cell carcinoma is that, not uncommonly, conventional oral SCC may show various histologic patterns in the same tumor: an exophytic component resembling papillary carcinoma, an extremely well-differentiated component with pushing border pattern of growth

tumor resembling verrucous carcinoma, or extensive areas where neoplastic cells are dyscohesive, exhibiting features seen in adenoid variant. How much of these changes are required to subclassify a tumor as one of the variants below is widely variable in the literature and different from one type to another.

5.2.5.1 Verrucous carcinoma (VC)

Verrucous carcinoma is a rare tumor in the United States, accounting for only 3 % of all oral carcinoma, occurs in elderly men [603], but comprises up to 16 % of OSCC in India [283]. VC has a strong association with chewing tobacco and has been well established from the original description of tumor in 1948 [282] and subsequently confirmed by other studies, but also betel nut has been implicated particularly in Asian patients [141, 284].

Despite earlier claims [19], it appears that there is no significant association with oncogenic HPV in two large recent series from India [286, 287] but is most likely due to tobacco chewing [283, 288] or betel nut consumption. Oral cavity is the most common site in head and neck area [285], with a predilection for buccal and gingival mucosa, buccal tumors accounting for 75 % of all head and neck cases [19].

Histopathologic appearance of verrucous cancer is well described and the characteristic features are relatively agreed upon: neoplastic fronds of squamous epithelium with extensive keratinization and no [19] or minimal evidence of atypia, dysplasia, or other mild malignant cytologic features and club-shaped rete stromal invasion with a pushing border usually eliciting dense lymphoplasmacytic stromal response (Fig 5.7). Despite the simple pathologic definition and easily recognizable features, this diagnosis may be extremely difficult or impossible in a small biopsy, since occasionally verrucoid lesions harbor conventional squamous cell carcinoma.

Even in excisional specimens, areas of cytologic atypia and/or angulated pattern of growth in an otherwise traditionally appearing verrucous cancer may create challenges in reaching a definitive diagnosis and excluding a conventional squamous cell carcinoma component. Some investigators accept that a degree of atypia restricted at the basal layer is expected in most verrucous carcinoma, albeit minimal [308], which is also this author's opinion; others accept "rare" mitoses or atypia [29] while, at the other extreme, some authorities describe it as lacking *any* "atypia, dysplasia, or other malignant feature" [19]. The latter definition appears to this observer as being extremely restrictive (probably a literal interpretation of the original description of Dr. Ackerman), and in my experience, nuclei are large, nucleoli are often present, and some dyskeratosis is seen.

Up to 20 % of VC are mixed or hybrid forms with a conventional SCC component [289]; this widely cited percentage is expected to vary, probably widely, with diagnostic criteria applied and individual threshold or philosophy. Extensive sampling is important to exclude a squamous cell carcinoma component since it is not uncommon to encounter foci of conventional squamous carcinoma in the excision of tumors diagnosed on biopsy as VC [283, 289].

Verrucous carcinoma may be multifocal, similar to proliferative verrucous leukoplakia (discussed in Sect. 5.3.4), which can progress to either conventional squamous cell carcinoma or verrucous carcinoma. There is a high likelihood that at least

some of the earlier verrucoid keratoses may represent in fact superficially invasive verrucous cancer. In one study of smokeless tobacco-associated carcinoma, 58 % of patients had a recurrence and almost half had concurrent leukoplakia, in which many verrucoid tumors were in fact conventional squamous cell carcinoma [290]. In a recent Indian cohort, one-third of VC patients had concurrent leukoplakia or submucosal fibrosis at the diagnosis and 68 % recurred [288].

Benign differential considerations include verruca vulgaris (exophytic pattern of growth exclusively) and squamous papilloma (fibrovascular cores, smaller cell size) [291], whereas other malignant tumors include papillary squamous cell carcinoma (discussed below) and carcinoma cuniculatum, an extremely rare entity with even more indolent biology than VC [292].

Verrucous hyperplasia is a controversial entity, some authorities considering it an early stage of verrucous carcinoma, a preneoplastic lesion in a continuous spectrum with verrucous cancer [19, 266, 293], while others avoided the term altogether replacing it with "papillary keratosis" [80].

Verrucous carcinoma is an indolent tumor with high potential for local recurrence but not for nodal metastasis particularly in the Western hemisphere [296, 297]. Local recurrences are common in all patients, but neck metastases are rarely reported even in large Indian series [283, 288]. It is unclear if a hybrid component is present in these metastatic tumors since some studies exclude these tumors [288] while others do not [283]. Moreover, exclusion of a SCC component would require assessment of the entire tumor, which may be difficult or unfeasible in large neoplasms in areas with limited resources or in laboratories with conservative sampling policies or philosophy. In another recent Indian series, hybrid carcinoma was reported in 51 % of excisional specimens and virtually all were missed on the biopsy [296]. In a large National Cancer Data Base analysis of 2,350 patients with head and neck VC, only 4.5 % had regional spread and 3.1 % had distant spread [285].

In summary, VC is relatively easy to diagnose on the excisional specimen with ample sampling, but it can be both over- and underdiagnosed on a biopsy, and definitive neck management decision should be postponed until microscopic evaluation of the entire tumor. Establishing consensus diagnostic criteria would be beneficial given the prognostic and therapeutic implications. How much cytologic and architecture deviation from the classical definition is acceptable for a tumor diagnosed and managed as pure verrucous cancer is currently unclear.

5.2.5.2 Papillary Squamous Cell Carcinoma (PSCC)

PSCC is a rare, well-differentiated tumor with papillary architecture more frequently encountered in the hypopharynx, larynx, and sinonasal tract [299]. PSCC is relatively rare in oral cavity, and in the largest series to date, the most common site of involvement was the larynx [298, 299, 302].

Definition of papillary squamous cell carcinoma, albeit relatively simple (a tumor with squamous differentiation and papillary architecture), is in fact quite controversial [306], given that many conventional squamous cell carcinoma may show focal papillary and/or exophytic protrusions (Fig. 5.7) [299]. At gross examination and at low power magnification under the microscope, the tumor resembles a

papilloma; however, marked cytologic atypia (carcinoma in situ) is present. By convention, even if no invasion is identified, this is not considered a carcinoma in situ. Determining the presence of stromal invasion is crucial given its prognostic significance but may be extremely challenging or impossible in the bioptic material. Luckily, many surgeons perform an excisional biopsy allowing examination of all or most of the tumor [298]. How much of a squamous cell carcinoma should have papillary architecture in order to qualify varies from one study to another. The strictest classification was from the AFIP investigators who required 70 % of the tumor to exhibit papillary growth [298]. Others included cases with a "significant" papillary component (not otherwise specified) [300], while some studies did not mention the specific morphologic criteria required for inclusion in this study other than papillary morphology [302].

The primary sites of the tumors described also vary from exclusively laryngeal [298] to predominantly oral cavity [301] to more recently oropharyngeal [302] or laryngeal-oropharyngeal [300].

The different inclusion criteria probably resulted in the variable outcome of this patients of the tumor which is thought to be indolent and having an excellent prognosis, superior to that of conventional squamous cell carcinoma: The disease-related mortality in laryngeal-rich studies ranged from 0 to 44 % [298, 299] begging the question if the same entity was described in these two large cohorts from the United States.

Similarly, the rate of human papillomavirus expression in this tumor is widely variable, but a consistent increase was noted throughout the studies: High-risk HPV types were detected in 0 and 29 % in older studies [298, 299] and increased to 33 % (larynx) [302] and 53 % overall [300] in most recent publications. In both latter studies, the prevalence of the oropharyngeal tumor was vastly superior to earlier studies, and high-risk HPV was detected in 85 % (p16+DNAish+) and 78 % (p16+mRNAish+), respectively, in papillary squamous cell carcinoma at this site.

It has been my experience that many of the HPV-associated carcinomas of the base of tongue and tonsil have an exclusively endophytic pattern of growth and, when sampled for a frozen section, exhibit markedly papillary growth. In more than one of these cases, the diagnosis of carcinoma in situ was offered intraoperatively to the frustration of surgeon who is sampling a large mass. In my opinion, these cases should not be classified within the papillary carcinoma spectrum. Particularly in the oropharynx, they may be just another histologic pattern that is already broad but rapidly expanding spectrum of HPV-related tumors, similarly to the basaloid variant (below). The situation of the HPV-associated tumors of the larynx and sinonasal tract is different since the viral presence has unclear clinical significance at this time [307]. There is no association of PSCC with laryngeal or sinonasal papillomatosis or schneiderian papillomas.

In summary, papillary squamous cell carcinoma is a rare variant of SCC with good prognosis when strictly defined; this variant is very rare in oral cavity and has a better prognosis than conventional squamous cell carcinoma particularly if no stromal invasion is present.

5.2.5.3 Basaloid Variant of Squamous Cell Carcinoma (BSCC)

BSCC is a variant squamous cell carcinoma of upper aerodigestive tract (WHO 2005) with a propensity for supraglottic larynx, hypopharynx, and proximal esophagus involvement that was described in 1986 [309]. In these locations, it is associated with an aggressive phenotype, confirmed in a large registry base study [312] but not in other [311]. In oral cavity, it is a rare tumor representing approximately 1 % of all squamous cell carcinomas [312]. Difficulties in diagnosis and critical importance of primary site and viral status may explain conflicting conclusions while analyzing the same registry [311, 312].

Basaloid squamous cell carcinoma is by definition a poorly differentiated carcinoma. Typical histology includes an organoid pattern of growth with nests of tumor surrounded by palisading basal cells; high nuclear-cytoplasmic ratio; lack of keratinization except the "abrupt" type, with occasional hyaline membrane deposition in the stroma; comedonecrosis; and a high mitotic grade (Fig. 5.6). It is currently unclear how much basaloid component should be present in a tumor with mixed histology. It is probably advisable to report any component and provide its approximate percentage, our preference for laryngeal tumors.

While differential considerations included high-grade neuroendocrine carcinoma, solid variant, or dedifferentiated adenoid cystic carcinoma, there is an increasing rate of human papilloma virus-associated oropharyngeal squamous cell carcinoma showing similar but not identical histologic features. Many of these tumors have been described as basaloid carcinoma although there are clear morphologic differences with the classically described hypopharyngeal and laryngeal tumors [313]. While some investigators classify the HPV-positive oropharyngeal nonkeratinizing SCC with basaloid-like morphology as basaloid squamous cell carcinoma variant [316, 317], it is probably safer and feasible [318] to distinguish the two categories given their morphologic, anatomic, and molecular differences. Moreover, despite the poorly differentiated morphology, HPV+ oropharyngeal SCC is known to have a much better response to radiation and overall improved survival [314, 315]. Mixing the two groups (smoking-related, p53-positive, HPV-negative laryngeal/hypopharyngeal carcinomas and HPV-related, P53-negative tumors occurring in the oropharynx of nonsmokers) may play a major role in the confusion existing in the clinical literature on the outcome of BSCC [313].

In our laboratory, we do not make a diagnosis of basaloid squamous cell carcinoma in the oropharynx, and we test all the extra oropharyngeal tumors (including oral tumors) with basaloid features to exclude HPV. We reserve the diagnosis of basaloid SCC for HPV-negative extra oropharyngeal tumors with classical morphology as originally described [309].

In a retrospective registry review of 119 oral BSCC reported in the English literature by 2013, it was not found to have a different clinical outcome from conventional SCC. Areas adjacent to the oropharynx, primarily oral tongue, floor of mouth, and the retromolar trigon are the most commonly involved oral sites in this recent review [320]. Most of these reported tumors were not tested for HPV, but 89 % of

patients were tobacco users [319, 320]. A recent database review identified similar outcomes with conventional OSCC [310].

Correctly identifying the site of the tumor by the surgeon and avoiding vague descriptions (i.e., "tongue") is important in this setting to exclude an oropharyngeal tumor and to direct the appropriate workup.

In summary, BSCC is usually an aggressive tumor in the extraoral sites, but currently many of these cases are likely oropharyngeal HPV positive and different in histologic appearance, biology, and outcome from the original description of classical BSCC. BSCC is rare in oral cavity and may have a clinical outcome similar to conventional SCC. Identifying tumors adjacent to the oropharynx to exclude local extension and HPV testing is important to distinguish the tumors with potentially favorable prognosis.

5.2.5.4 Spindle Cell (Sarcomatoid) Carcinoma

Spindle cell carcinoma (SPSCC) is a rare variant of squamous cell carcinoma, defined by its malignant mesenchymal phenotype. It is associated with an invasive or in situ squamous cell carcinoma in most cases [18, 19, 308]. Other designations (sarcomatoid carcinoma, carcinosarcoma, pseudosarcoma, pleomorphic carcinoma) were historically used [18].

SPCC presents in patients with typical etiologic factors including smoking and alcohol, but cases occurring after radiation were also reported [18]. Etiology of these tumors has been debated in the past; however, it is clear now that the mesenchymal component is clonally derived from the squamous epithelium [321].

Most of the clinical and pathologic experience was gained from laryngeal neoplasms, the most common primary site for this variant [322, 323]. SPSCC are rare in oral cavity (approximately 1 % of all squamous cancers); they present as a pedunculated or sessile polyp or, less likely, as an ulcerated-infiltrative mass and involve predominantly the lower lip, tongue, and alveolar ridge [324, 325]. Situation is different in other regions where tobacco chewing is prevalent: for instance, in a recent Indian study, oral cavity was the most common head and neck site and buccal mucosa the most common oral subsite for SPSCC [328]. Radiation exposure was found to play a role in 17 % of the Western patients, mostly at laryngeal sites [19], but in none of the Indian patients, two-thirds of whom have oral site involvement [328].

Histologically, these tumors consist predominantly or exclusively of a spindle cell malignant proliferation, but approximately 5 % heterologous elements are present including osseous and cartilaginous differentiation [19, 322].

If the squamous component or supradjacent dysplasia is identified, diagnosis is immediate, and no further studies are required; however, often in small bioptic samples, immunohistochemical studies will be required and reveal expression of various keratins with a cumulative expression in 68 % of tumors; myoid markers can also be positive in a high percentages of cases [322], and therefore, the differential diagnosis includes various sarcomas as well as benign fibroblastic proliferations and superficially sampled tumors. Absence of cytokeratin does not exclude the diagnosis [322, 326, 327], and conversely, true sarcomas can express epithelial markers.

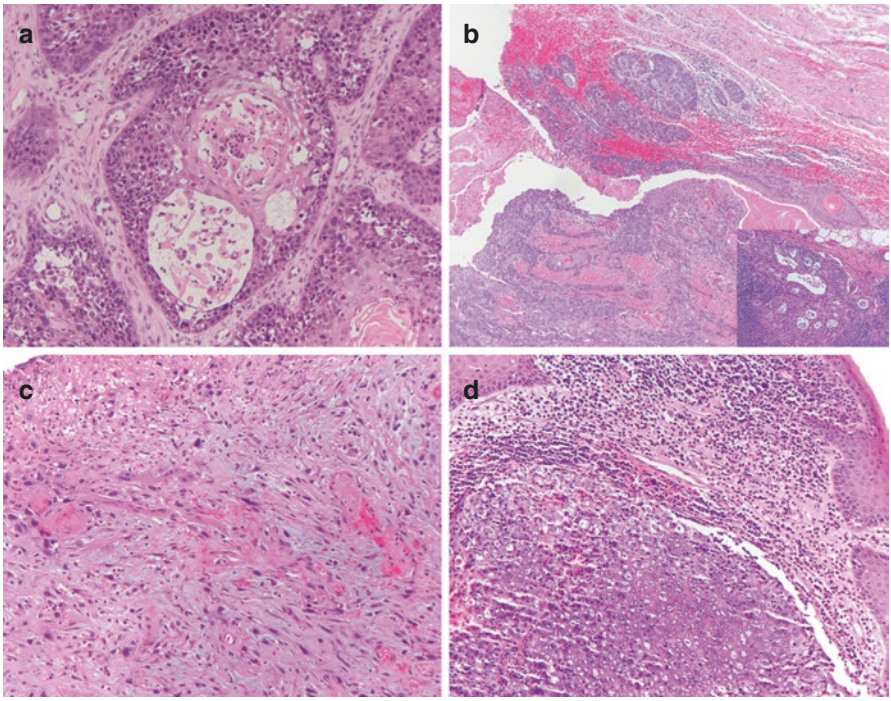

Fig. 5.10 Variants of OSCC. (**a**) Adenoid (pseudoglandular) carcinoma with gland-like formation due to tumor cell acantholysis. (**b**) In contrast, adenosquamous variant exhibits dual differentiation: a keratinizing squamous component (*right lower*) and a true glandular component (*left upper*); the latter developed nodal metastases (*inset*). (**c**) Spindle cell (sarcomatoid) squamous cell carcinoma of the oral tongue is often polypoid with surface ulceration (*left upper*). (**d**) Lymphoepithelial carcinoma of oral cavity is extremely rare and this morphology more likely to be encountered adjacent to the oropharynx where is usually HPV-related (unlike in the nasopharyngeal site, where is EBV-associated), as was the case in this tumor involving the retromolar trigone and pharyngeal tonsil

Prognosis is somewhat controversial, but generally oral SPCC fare worse than laryngeal ones [19], 55 % of patients died of diseases in average in less than 2 years [324]. Superficial depth of invasion and lack of cytokeratin expression in the mesenchymal component have been reported to be associated with a better outcome in larynx; however, data in oral cavity SPSCC are too limited for a definitive conclusion.

5.2.5.5 Adenosquamous Carcinoma

Adenosquamous carcinoma (ADSCC) is a rare, aggressive variant of squamous cell carcinoma with dual differentiation, squamous and glandular, described in 1968 [329]. Slightly over 100 cases were reported to date in oral cavity [330].

Two distinct components define this variant: a superficial, keratinizing squamous cell carcinoma component usually predominate and a glandular component (adenocarcinoma) has a deeper localization [331]. While there is usually a clear demarcation between the two components, they may intermingle.

In situ carcinoma or high-grade dysplasia is usually seen and would support the currently most accepted histogenetic hypothesis of its origin in a multipotent basal cell of surface epithelium, although the issue is not entirely settled. A recent case arising in lingual leukoplakia with a glandular component present at the microinvasive stage, subsequently progressing into typical biphasic ADSCC, would further support a surface epithelial origin of this tumor [332]. Unfortunately, other recent series still include primary tumors of major salivary glands with this phenotype which would not fulfill the currently accepted WHO definition of the variant [330].

Oropharyngeal but not oral tumors can be associated with human papillomavirus in a minority of cases, when the prognosis is apparently better, although too few cases have been reported for a definitive conclusion [333].

The major differential consideration includes mucoepidermoid carcinoma with which this rare tumor has been undoubtedly confused in the past [330]. Most authorities require the presence of an in situ component and keratin pearl formation for this diagnosis, reserved for tumors arising from surface mucosa [334]. The two components are labeled distinctly with squamous (p63, high molecular weight keratin) and glandular (intracytoplasmic mucicarmine, CEA, CK7) markers [331, 332]. Presence of glandular markers is essential in distinguishing these variants from the acantholytic (adenoid) pattern in some cases or small samples.

Prognosis in the three largest series to date has been invariably worse than conventional squamous cell carcinoma, confirming earlier small series and case report findings [335–337] and much worse than mucoepidermoid carcinoma, making this distinction clinically relevant. Overall, only slightly over 100 cases have been described to date in the head and neck mucosal sites [330, 331, 333, 334] with neck metastasis in approximately 70 % and distant metaphysis in a fourth of cases. The 5-year survival is estimated at 15–25 % [297, 338]. Therefore, an aggressive management may be indicated in this rare variant including in oral sites.

5.2.5.6 Acantholythic Squamous Cell Carcinoma

Acantholythic (adenoid, pseudoglandular SCC, angiosarcoma-like, pseudovascular, adenoid) SCC was described and is most commonly encountered in cutaneous squamous cancer but is recognized as a histopathologic variant of head and neck mucosal neoplasms by WHO classification [297].

As previously alluded, it is unclear if this is a clinicopathologic entity or merely a histologic pattern, in our experience not uncommonly seen in many mucosal conventional keratinizing squamous cell carcinomas as described by others [63, 308]. Currently there is no minimal requirement for these morphologic changes to classify an otherwise unremarkable squamous cell carcinoma; therefore, existing data are restricted to case reports or very small series [339–343].

In head and neck areas, sun-damaged skin, including the lower lip vermilion, is the most common site and was associated with good outcome [344] in contrast with the intraoral tumors, which showed a more aggressive course [19].

Its significance resides in the superficial morphologic overlap with adenocarcinoma, adenosquamous carcinoma, and angiosarcoma which is not difficult for the experienced observer [63]; in selected cases, immunohistochemical studies will quickly settle the issue.

Due to its rarity in oral mucosa, unclear definition, and reporting bias, the suggested aggressiveness [345, 346] attributed to this morphologic pattern is difficult to confirm at this time.

5.2.5.7 Lymphoepithelial carcinoma

Lymphoepithelial carcinoma (lymphoepithelioma, undifferentiated carcinoma, nasopharyngeal type) is common in nasopharynx in Asian patients where it is EBV-related but is extremely rare in oral cavity with only around 20 case reports existing to date, and is somewhat more common in oropharynx [347].

Most extra-nasopharyngeal lymphoepithelial carcinomas are considered aggressive diseases with propensity for regional and distant metastasis [348]. However, intraoral tumors can be EBV related in patients of Asian descent [604] but usually not in Caucasian patients [352, 353].

The predilection of lymphoepithelial-like tumors for the oropharyngeal area (tonsils, base of tongue) from all non-nasopharyngeal head and neck areas was noted [347, 351, 604]. All these tumors were Epstein-Barr virus negative, and the reason for this propensity may have been recently revealed: these histologic changes are focally but not uncommonly present in HPV-positive oropharyngeal tumors and it appears that some tumors may have extensive or complete lymphoepithelial morphology [352]. Since these changes can be focally present in many oropharyngeal HPV- related SCC, these tumors should not be probably grouped based on their histomorphologic appearance but based on their viral status. Rare case reports of minor salivary gland lymphoepithelial carcinoma exist [349].

In summary, oral lymphoepithelial carcinoma is extremely rare; when occurring in oropharynx or adjacent oral sites, testing for both human papilloma virus and Epstein-Barr virus is recommended. The data are scant for a conclusion regarding their biology in the oral cavity sites.

5.2.6 Pathology Considerations of Nodal Disease Assessment

Nodal involvement by OSCC is the single most important adverse prognostic factor, correlating with increased regional recurrence and metastatic potential in univariate and multivariate analysis and reducing the overall survival by half [120, 353–355]. Interestingly, a recent multicenter retrospective study found that the negative survival impact was preserved even in patients with clinical positive lymph nodes even if not confirmed by pathologic examination (cN1, pN0) [149].

Oral cavity cancer relatively predictably metastasizes to levels 1–3, usually ipsilateral, and its patterns of dissemination may vary with the primary site [356–358]. Skipped metastasis or bilateral metastasis from oral cancer, unlike oropharyngeal, hypopharyngeal, or laryngeal cancer, is relatively uncommon [357], mostly seen in midline tumors and tumors of the tongue [151].

Important pathologic prognostic indicators are size of the metastatic deposit, size of the involved lymph node (not identical measurements), number and level of involved lymph nodes, presence of extracapsular extension, soft tissue deposits, and pathologic total stage [151].

Specimens should be received labeled from the operating room, type of procedure clearly described, and nodal groups appropriately labeled and preferably separately submitted by the surgeon according to the consensus statement of American Head and Neck Society [360]. In the absence of anatomic landmarks, only an approximate orientation can be made by the surgical pathologist in dividing levels 1–5. It is practically impossible to precisely distinguish between the three jugular groups (levels II, III, and IV) or the sublevels of levels I, II, and V, and the pathologist would have to approximate these levels in the unlabeled en bloc specimen.

The *number of involved lymph nodes* was shown to be an adverse prognostic finding in multiple studies [120, 125, 353, 354, 361]. Two key determinants are the type of surgical procedure and pathology dissection completeness [362, 364]. Individual variability of both techniques within and between centers, as well as strict definition of particular circumstances such as matted lymph nodes, may account for the difference of lymph node yield and outcome [357]. In retrospective studies, central pathology review and assessment of lymph node status blinded to clinical information, outcome, and previous pathology report would be ideal, but these cases are the exception in the literature [357, 365].

An increased number of *total* lymph nodes harvested has been shown to increase probability of positive finding by the pathologist [362, 363] and is an independent prognostic factor [366]. No strict guidelines for minimal lymph node numbers to be harvested exist for neck dissection: some guidelines suggest a minimum of six and ten lymph nodes for selective and radical neck dissection, respectively [11]. This is a very modest target and, in our experience, more lymph nodes can (and should) be found in the neck of untreated patients, as shown in several studies which reported a yield of 5–11 nodes per level [357, 367, 368, 381]. Probably a better minimum total lymph node yield should be 18–20 as suggested in two previous studies [362, 366]. A recent multicenter study published by the International Consortium for Outcome Research (ICOR) confirmed the strong prognostic value of lymph node yield and defined the minimum acceptable yield as 18 lymph nodes in an elective neck dissection [369]. If the yield is low, the lymph node density (ratio between positive and total sampled lymph nodes) was suggested as a good predictive alternative [365], recently validated in a multicenter retrospective study by ICOR [364]. While good dissection technique is sufficient in detecting all or most nodes, ancillary solutions exist and can be used to enhance or facilitate detecting additional nodes and potentially upstage the patient [370] since a third of the dissected nodes can measure less than 3 mm [381]. These latter investigators found an average of 50.4 lymph nodes/neck dissection and suggested that all tissue including fat be submitted for microscopic examination to capture all micrometastasis and soft tissue deposits. This technique, however, entails a significant resource commitment which may not be widely available and, to my knowledge, no other institutions use it in their routine clinical practice.

All negative nodes should be entirely evaluated microscopically, while one section suffices for grossly positive nodes [11, 359] but more could be submitted if macroscopically the extracapsular extension is not evident. Detection of micrometastases is possible using conventional histologic assessment [152] or employing immunohistochemical studies (see below).

Size of the largest deposit of metastatic tumor and not of the involved lymph node should be measured by the pathologist and recorded in the pathology report [11, 12, 238].

The size-dependent classification of metastatic disease was recently reviewed [151]: Isolated tumor cells are defined by UICC/TNM system as a group of solitary or small collections of neoplastic cells in nodal parenchyma or sinuses measuring less than 0.2 mm or maximum 200 cells [238]. Significance of isolated tumor cells in OSCC is currently unclear, and using ancillary methods or specific detection means is not recommended for staging purposes [11, 12].

Micrometastasis is defined as single or multiple deposits confined within the lymph node measuring 0.2–2 mm in greatest diameter (Fig. 5.11). Significance of micrometastasis and their impact on prognosis is not entirely clear [371–373], but it has been studied more extensively with the sentinel lymph node biopsy – see below discussion – and it appears that the patients with pN1(mi) have an intermediate prognosis between pN0 and pN+ [359, 374]. Conventional metastasis is defined as any metastasis with a diameter larger than 2 mm. An occult metastasis is defined as isolated tumor cell, micrometastasis or conventional metastasis in a cN0 neck.

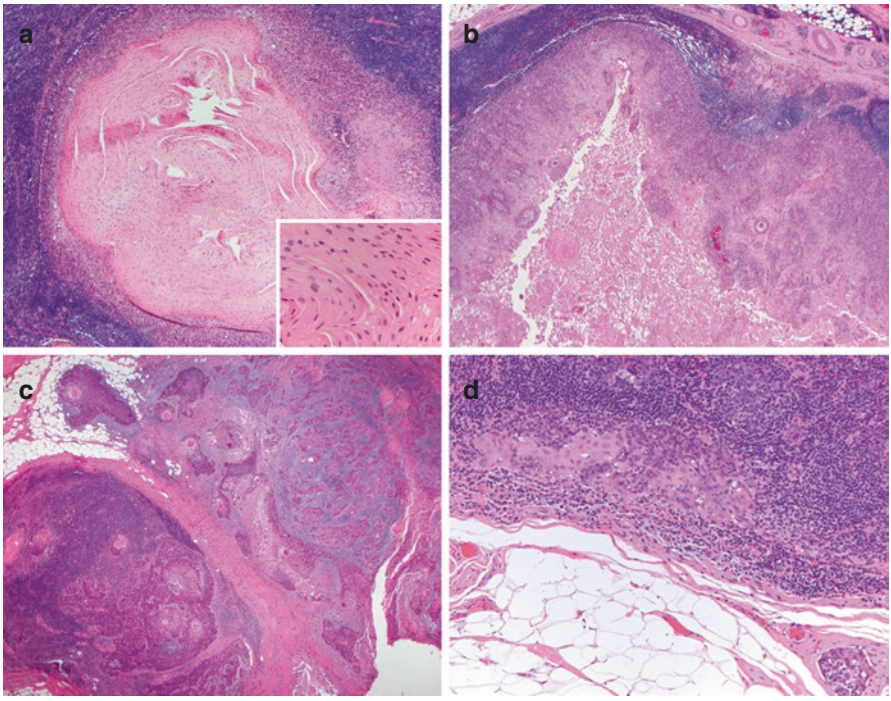

Fig. 5.11 (**a**) Small metastatic deposit of well-differentiated squamous cell carcinoma with extensive mature, bland-appearing keratinization (*inset*). (**b**) This large metastasis shows cystic degeneration. (**c**) Several metastases in a small (3 mm) lymph node with extracapsular spread and microcalcifications (*right lower*). (**d**) A single micrometastasis (1.1 mm) was identified only in a nonsentinel but not in the sentinel submental node in this 58-year-old man with anterior floor of mouth cancer. Note the focus of tumor in the adjacent afferent lymphatic vessel (*right lower corner*)

Extracapsular extension or spread (ECS) is a major independent prognostic factor for disease-specific and overall survival in both localized and advanced oral cancer [118, 142, 194, 214, 356, 365, 369, 375], a predictor of regional recurrence, and constitutes an indication for postoperative radiation [188].

ECS is defined as extension through the nodal capsule in the adjacent soft tissue and can be either minimal, detected at microscopic evaluation, or grossly, when clinical, macroscopic, or radiologic evidence of adjacent soft tissue involvement or matted nodes are present, [15]. Distinction between microscopic and macroscopic ECS, as well as recording the distance of the microscopic tumor cells from the capsule, is considered currently optional in pathology reports [11, 12, 151, 376].

Capsule thickening and stromal changes outside the capsule were proposed to represent early ECS [151, 233]. There is a wide variation of the rate of identifying this finding in the literature, likely responsible for the often conflicting results [377, 378]. Modern imaging was reported to have only 50 % sensitivity for ECS detection [379].

Pathologic challenges in identifying ECS occur with tumors involving the hilum, tumors with subtotal replacement of the lymph node architecture but lacking desmoplastic reaction, or tumors bulging outside the lymph node outline. Some [376] but not all [380] authors consider at least some of these findings as being positive. A high interobserver variability was reported among pathologists [380]. Currently there is a need for consensus and/or guidelines given the clinical impact of ECS.

In oropharyngeal SCC, unlike oral tumors, the prognostic ECS significance is diminished in HPV-associated tumors [206, 384], but is retained in HPV-negative neoplasms [151].

Despite its significance in oral cancer, with direct prognostic and therapeutic consequences, the presence of ECS was found to be missing from 27 % of pathology reports [234]. ECS recording is mandated for oral cancer synopsis, but distinction between the micro- and macro-ECS is not currently required [11, 12].

Soft tissue deposits are foci of tumor involving skeletal muscle or fat, reported in up to 11 % of all HNSCC neck dissections, and are adverse prognostic findings [197, 368, 381, 382]. They are counted by convention as positive node with ECS [15]. Their definition and pathogenesis currently needs refinement since most adipose tissue is not evaluated microscopically in routine clinical practice, and therefore this finding's true incidence is unknown. Most published experience comes from a single group [368, 381–383, 387], and standardization of evaluation and reporting would be desirable.

Sentinel Lymph Node Biopsy (SLNB). Predictable nodal drainage and predominant lymphatic spread of this neoplasm which rarely skips nodal stations, together with well-mapped lymphatic drainage of the neck, make SLNB an alternative or additional staging procedure for tumors of this site [384].

Advantages include accessibility, lower morbidity, and comparable accuracy with selective neck dissection [386, 392]. Detection of occult metastases which occur in up to one-third of patients with cN0 neck [384, 387] avoids overtreatment of the negative neck in early oral cancer.

Several European centers have used SLND extensively in the last decades, and guidelines for the technical aspects to ensure uniform practice were recently introduced [388]. The procedure was validated in the United States in a prospective multicenter study [389], and SLNB was recently incorporated in various clinical guidelines [188, 388].

Clinically, SLNB is indicated only for OSCC with T1/2 and cN0 neck and is generally contraindicated in clinically positive neck. Methods used include preoperative lymphoscintigraphy with gamma probe detection with or without intraoperative blue dye, the former preferred in most single center cohorts and multicenter studies [384, 387–389].

Successful detection of SLN was reported in >93 % of cases [388, 389] better in some subsites with more predictable spread. Variable numbers of SLN are detected, in average 2.5 [384], depending on operator experience. SLNB has excellent accuracy, in two recent meta-analyses pooled sensitivity of 0.93–0.94 and negative predictive value of 0.95 (range, 0.88–1) being reported [384, 390].

Best usage for SLNB is currently in T1–T2 of oral cavity and accessible oropharynx areas with a clinically negative neck. Level 1 proximity to FOM is likely the technical cause of lower predictive value in this location due to "shine through" artifact [387].

Pathologic Considerations of Sentinel Lymph Node Biopsy SLN evaluation has been extensively described in breast cancer and melanoma. The proposed protocols for OSCC SLNB follow those established at other sites [388, 391]. In brief, the sentinel node(s) identified by the radiotracer and/or dye is sliced along the hilar axis of the largest diameter; depending on SLN(s) size, it is further sectioned or entirely embedded en face; alternate sections for hematoxylin and eosin (HE) and IHC studies are cut at 150 μ; and IHC staining is performed only in HE-negative cases [388, 391].

Regarding the sampling methodology and number of levels to be examined, current European guidelines lean toward an exhaustive sampling of HE-negative nodes [388], method that can potentially render up to 200 slides for three sentinel nodes, which would be extremely labor intensive and impractical to incorporate in routine practice [391–393]. Recent studies failed to identify additional micrometastases or isolated tumor cells with serial step sections [394, 395, 410], while others found additional micrometastases but without detecting false-negative necks [396]. There is evidence that metastases concentrate in the hilar plane of SLN [397] and some investigators recommended a limited, gradual sampling from this plane toward the outer aspect of the node [394, 398].

In many single cohort studies, the pathologic examination methodology was described only briefly or was unclear but appear that 15/21 (71 %) used histologic exam enhanced by IHC [384]. Some significant differences exist even in multicenter study design regarding histologic evaluation [388, 389]. Immunohistochemistry upstaged the neck in approximately 20 % of cases [384] and is used by most centers. Its use is restricted to HE-negative sentinel nodes [388, 391] and deemed mandatory in most recent recommendations [392].

Frozen section (FS) in SLNB has a low sensitivity and is relatively less studied than in breast cancer. The false-negative rate using frozen section is high for both oral [399] and oropharyngeal cancer [400], detecting only half of the positive SLN when compared with step serial sections/IHC method. Other groups reported better sensitivity [401, 402], but there is certain variation among different techniques used locally. Even in the same center experience, the SLNB FS sensitivity decreased with longer follow-up [401, 403]. No current recommendations for FS usage in the SNB for oral cancer exist [388, 392], but adjustment to local expertise and resources is essential.

Several pathologic studies validated the procedure and described a detailed methodology [391, 395, 408, 404, 405]. All nodal tissue harvested through sentinel lymph node procedure needs to be microscopically examined. However, over a thousand tissue sections can be obtained from a 5 mm thick node, given the usual 4–5 μm thickness section employed in clinical practice. Lymph node is sectioned in several 2–3 mm slices from the hilum outward. The necessity of multiple 150–250 μ serial sections and tissue exhaustion was questioned, and it may be impractical as discussed earlier. Adding immunohistochemistry to the intervening sections increases the detection of isolated tumor cells and micrometastasis primarily [388, 399, 400].

Despite significant progress in the field, imaging techniques show limitations in detecting micrometastasis and isolated tumor cells compared to histologic evaluation with or without histochemical studies [406].

The micrometastases are staged as pN1; there is currently no agreement on the clinical significance and reporting of isolated tumor cells. Molecular staging using PCR may prove valuable but is currently not used in clinical practice [11, 407]. Developing specific primers to exclude false-positive results due to frequent benign epithelial inclusions will be essential for successful implementation [391]. SLN reporting should follow existing current synoptic guidelines [11, 15, 12, 238, 388].

SLNB procedure has been established, but protocols need to be in place in the centers that would adopt it. Surgeon's individual experience is a factor [389], and the local SNB standard operating procedure should be implemented, with clearly delineated methodology, pathologic processing, and turnaround time expectations – all defined by the center multispecialty team [388]. Current histopathologic processing protocols may not be feasible when local resources are limited, and more limited sampling may suffice based on lymphatic drainage models and empirical data to date [395, 398, 405]. While frozen section may be incorporated in the HNSCC SNB protocols, current data are conflicting, and further better controlled studies are needed for a definitive conclusion. In sum, SNB is an accurate validated staging procedure for oral cancer with comparable accuracy and lower morbidity than elective dissection. Surgeons and pathologists are responsible of establishing a standard operating protocol, and close collaboration is essential for successful clinical practice implementation of this powerful staging modality.

5.2.7 OSCC in Specific Circumstances

5.2.7.1 Human Papillomavirus Role in Oropharyngeal Squamous Cell Carcinoma

Oropharyngeal carcinoma has been increasing in prevalence and HPV-related tumors displaced the conventional SCC at this site in the United States, Canada, and Europe, accounting for approximately 70–80 % of all oropharyngeal SCC (OPSCC) in recent epidemiologic studies [408, 409]. The increase has been attributed to a change in sexual behavior, recently confirmed in a large study [410].

Oropharyngeal HPV+SCC occurs in younger, nonsmoker men (fifth to sixth decade), has a typical nonkeratinizing phenotype, higher radiosensitivity [314, 315], and overall better prognosis than conventional SCC despite its propensity for early lymphatic spread.

Many of these patients present with large, often cystic metastases and comparatively small primary tumors, and therefore some are often deemed clinically tumors of "unknown primary" [411, 412]. For this reason, we test all unknown primary squamous cell carcinomas for HPV infection in our practice. However, the spectrum of HPV+ OPSCC is quite broad and mixed (hybrid) or predominantly keratinizing tumors are well described. In addition, as previously discussed, papillary, adenosquamous, neuroendocrine or lymphoepithelial (Fig. 5.10) phenotypes have been reported with unclear impact on patient outcome. While most HPV+ OPSCC are nonkeratinizing, we and others [417, 419] have seen HPV-related well to moderately differentiated keratinizing SCC at this site. Therefore, all oropharyngeal cancers should be tested for HPV regardless of histologic phenotype.

Most HPV+ OPSCC tumors have HPV16 type which through E6 and E7 oncogenes deregulate the retinoblastoma pathway and upregulate the p16, the latter widely used as a surrogate marker for HPV. However, its use should be made with caution since none of the several tests available today in the clinic are sufficiently specific in isolation [413].

Various testing strategies have been proposed, but currently no definitive consensus exists on the optimal combination. p16 is probably the most widely used marker, being inexpensive and highly sensitive, but its specificity is around 80 % when compared with E6/E7 mRNA, the gold standard [413, 414, 425].

DNA studies are either PCR-based or fluorescence in situ hybridization; the first are much more sensitive but less specific, and viral infection unrelated to the tumor ("passenger infection") can be detected. The latter are the preferred one by most pathologists allowing the visualization of viral particles in the neoplastic cells and can include one (HPV 16), two (HPV 16,18), or multiple probes, various cocktails against multiple high-risk HPV types (HR-HPV) being commercially available.

Presence of viral DNA does not prove causality, and currently RNA studies are considered the gold standard to establish "driver infection" and include RT-PCR (limited by technical challenges in RNA extraction from paraffin embedded tissue)

and mRNA in situ hybridization which has become commercially available in the United States in recent years and validated by two groups [415, 416].

p16 is arguably the most utilized test in clinical practice. Both cytoplasmic and stromal nuclear staining in at least 50–75 % of tumor cells is required. In the presence of typical morphology (nonkeratinizing, basaloid-like), strong, diffuse expression of p16 is a reliable marker for transcriptionally active HR-HPV. There is some evidence that p16-positive tumors lacking any other DNA or RNA evidence of HPV infection are associated with favorable prognosis [420]. While p16 sensitivity and specificity for HR-HPV is 97 % and 84 %, respectively, ISH HPV16 has 88 % sensitivity and 95 % specificity [414]. Also, the degree of interobserver variability is lower for p16 than ISH as we and others noted [414, 417, 418], and different probes from various manufacturers have different performance as we noted in our clinical practice and others documented in a comparative study [418].

Currently there is no consensus on best HPV testing strategy [426], but most centers use a multi-testing methodology, usually p16 and DNA in situ hybridization.

5.2.7.2 Human Papillomavirus Role in Oral Squamous Cell Carcinoma

Human papillomavirus-associated squamous cell carcinomas have been variably detected at other head and sites, particularly sinonasal [307] and oral cavity; in the latter, high-risk human papillomavirus DNA-weighted prevalence was 20.2 %, 23.5 %, and 39.9 % [423–425] in several meta-analyses of numerous earlier studies using various DNA techniques.

In contrast, *oncogenic* HPV is rarely detected in oral SCC when tested with newer validated methods assessing transcriptionally active virus in the range of 0.1–5.9 % [415, 416, 421, 422]. Of note, expression of p16 protein or DNA detection is now considered to significantly overestimate the viral importance since up to half of these cases do not transcribe mRNA E6/7 [414, 421]. Positive predictive value of p16 expression for HPV infection is much lower in OSCC than in OPSCC [421], and it should not be used in isolation as a surrogate marker for viral infection at this site.

Similarly, detection of viral HR-HPV DNA detected by PCR may simply reflect prior exposure to the virus of oral mucosa without oncogenic potential since only half of the cases with HPV DNA also expressed E6/7 mRNA [421].

HPV is not increased in young, nonsmoker patients with OSCC [422, 425], in contrast with OPSCC where almost all nonsmoker patients have HPV+OPSCC. The vast majority (95 %) of HPV-associated OPSCC is related to HPV16, whereas in OSCC other HPV types constituted 38 % of all positive cases [421]. HPV-associated OSCCs were most common in the floor of mouth and had a disproportionate amount of poorly differentiated tumors with basaloid-like phenotype in one study [421]. A survival benefit as seen in HPV+ OPSCC has not been demonstrated in OSCC patients, and its clinical significance is currently unknown.

Consequently, routine testing of OSCC for HPV is not currently recommended given that, when tested with validated methods assessing the transcriptionally active status of viral infection, only in less than 6 % of OSCC cases oncogenic virus is present [421, 426] and has clinical unclear significance.

5.2.7.3 Squamous Cell Carcinoma in the Younger Patients

There is recently growing epidemiologic evidence of an increasing trend of oral squamous cell carcinoma (OSCC) in younger patients (<40 years old) reported in multiple studies from Europe and the United States [427–429], but this seems to be a global phenomenon with similar findings described in Brazil, India, and other countries [13, 430, 431].

Oral SCC incidence in young patients varies with the definition (30, 40, or 45 years cutoff have been used) and is currently estimated at 5 %, but rates up to 14–18 % have been reported in some referral centers [230, 427, 432].

Many patients under 40 have minimal or no exposure to conventional risk factors (smoking, alcohol) or human papillomavirus. Patients of age 40–45 may have similar histories or belong to the conventional risk factor group [433], but the current evidence for increased incidence in young nonsmoker women is strong [429].

Genetic predisposition may play a role in very young adults or pediatric group, SCC occurring in the latter being associated either with familial history of cancer [430] or with various inherited or acquired immunodeficient conditions including xeroderma pigmentosum, Fanconi anemia, and post-bone marrow transplantation [62, 436]. Fanconi anemia gene deregulation has been reported in some of these rare occurrences [433, 437]. Distinctive molecular alteration patterns in the young compared to older patients were lower frequency of 3p and 9p loci changes [434] and higher aneuploidy [435], suggesting, together with lack of tobacco and alcohol exposure in many young patients, a biologically distinct tumor from smoking-related conventional SCC [436]. The precise causes and mechanisms involved are unclear at this time [440], but likely HPV does not play a role [422].

These tumors involve predominantly the oral tongue [428, 436], more commonly the anterior oral sites compared to older patients [432], and are histologically variable, but almost all exhibit conventional keratinizing SCC histology as depicted in Figs. 5.1 and 5.7. Most studies detected no significant histologic or pathologic risk factor difference when compared with similar tumors occurring in older adults. Few studies reported more aggressive histologic patterns compared with older patients [438], higher nuclear aberrations but lower proliferation index [154], and a trend for poorly differentiated tumors [230] in the young patients (Fig. 5.7), but many other authors reported no histopathologic difference among the two age groups [439, 441].

Prognosis of OCSCC in young patients is also controversial: recent studies showed a more aggressive disease with advanced stage at presentation [230, 442], while others failed to identify an outcome difference [436, 443]. As recently pointed out, most of these single center studies are underpowered for multivariate analysis, and only a multicenter study may definitively answer these questions [436].

In summary, there is evidence of increasing incidence of oral cancer in young nonsmoker women, particularly in the developed world where overall smoking rates have decreased. Early detection is important for curative treatment.

5.2.7.4 Squamous Cell Carcinoma in the Immunosuppressed Patient

These tumors have had recently increased prevalence due to therapy availability and medically induced immunosuppression [444, 526] such as bone marrow transplant. Patients with graft-versus-host disease treatment are at highest risk for developing OSCC (Fig. 5.2) [446].

The underlying mechanism is likely related to a decrease in cellular immunity; patients with decreased regulatory T cells were recently shown at risk for developing multifocal disease [447].

These tumors are more aggressive and therapy resistant, with poorly differentiated histology and/or aggressive pattern of growth (Fig. 5.2). Several studies showed association with decreased disease free and overall survival, and therefore early biopsy and more aggressive surveillance may be recommended particularly in the graft-versus-host disease (GVHD) setting since OSCC can mimic GVHD lesions [448].

5.2.8 Diagnostic Issues, Differential Considerations, and Mimics of Oral Squamous Cell Carcinoma

OSCC diagnosis is usually straightforward, but can be problematic or early detection opportunity missed due to very good maturation which may mimic benign keratosis in superficially sampled well (or very well)-differentiated tumors. Most pathologists are trained to "err on the side of the benign", certainly a laudable and prudent approach when one envisions life-altering consequences of oral cancer diagnosis. However, in our experience, most errors in general practice are due to underrecognition of malignant changes, or undergrading of dysplasia (see below) leading to delay in the definitive cancer diagnosis or disease progression risk underestimation. This delay in making the diagnosis or identifying progression (and not the overdiagnosis as pathology folklore wisdom has taught many generations) was shown recently to constitute the bulk of medicolegal cases in the United States. Pathologists are rarely sued, but when this happens it is for failure to diagnose [449].

Major diagnostic discrepancies are generally defined as diagnostic changes with significant influence on patient management and/or prognosis. The area of head and neck is considered a "high-risk" diagnostic site with major discrepancies reported at re-review and second opinion in the 7–16 % range [450–454]. While this incidence may appear relatively high, it is important to note that the internal consultations among pathologists are an important mechanism of quality control in tertiary care centers and the number of potential diagnostic errors thus preempted by the in-house review even in large centers is essentially unknown. The most common lesions prone for a major discrepancy in oral pathology practice include salivary gland tumors, squamous dysplasia, carcinoma and their mimickers, and odontogenic cysts [454], but there is certainly a wide variation based on pattern and reason for referral. Obviously, the type and the frequency of specific diagnostic discrepancies will be different where mandatory pathology reassessment is an internal policy, such as most cancer centers or large referral centers in the United States [450],

compared to the cases retrieved from personal consultation files of a pathology authority, which may be biased on the consultant expertise and record [454]. Prospective consultation (so-called double reporting) is desirable, whereas second opinion is retrospective in most cases. The most common diagnostic problems in head and neck pathology have been recently reviewed [455].

A broad gamut of difficulties can occur and pose significant challenges, particularly when only a small biopsy is available, and may result in diagnostic delay, overtreatment, or undertreatment. Some were already discussed earlier under various subtypes or sites of oral cancer where they are most likely to be encountered; many are described in most textbooks or reviews and will be only briefly mentioned below; and other pitfalls recently encountered in our experience may be underrecognized by the clinicians and pathologists alike. If the list of these difficulties has not drastically changed in the last three decades [456, 457], our means to resolve them have expanded significantly, with the technical progress and broad availability in immunohistochemistry and cytogenetics, as well as molecular-genetic studies. It is important that the clinician remembers that, for all various differential considerations and diagnostic pitfalls listed below, frozen section artifact and previous radiotherapy of the area biopsied significantly compound the difficulty of reaching the correct diagnosis. In a cancer center setting, encountering both these challenges in the same case during an intraoperative consultation is not unusual. As will be discussed, radiation atypia may result in the benign versus malignant distinction being extremely difficult even when permanent sections are examined, let aside on frozen section slides, and the surgeon needs to be aware of this limitation.

Finally, the importance of clinicopathologic correlation cannot be overemphasized. A concerning histologic finding for the pathologist may have an immediately apparent clinical explanation (local trauma, bisphosphonate-related osteonecrosis) and vice versa.

5.2.8.1 Oral Squamous Cell Carcinoma Versus Other Cancers

Distinguishing squamous cell carcinoma from other malignant neoplasms is usually straightforward but in several circumstances it may be difficult.

Salivary gland tumors – mucoepidermoid, myoepithelial, and salivary duct carcinoma can all have a squamoid appearance or exhibit complete squamous differentiation which, particularly on small biopsy or fine needle aspirate, may closely resemble squamous cell carcinoma. Moreover, the overlap is not only morphologic but also immunophenotypic since the first two tumors share several markers (p40, p63, CK5/6, high molecular cytokeratin) with OSCC.

Many salivary gland neoplasms have a myoepithelial component or differentiation. Carcinoma ex-pleomorphic adenoma and pleomorphic adenoma can have extensive squamous differentiation, and myoepithelial clear cell carcinoma can show squamous metaplasia [458, 459] and exhibits squamous immunoprofile. We have encountered in our practice myoepithelial tumors of salivary gland origin misinterpreted as squamous cell carcinoma as recently reported [458]. Expression of myoid markers and presence of plasmacytoid cells, myxoid stroma, and/or ductal elements are clues to the correct diagnosis and tumor origin. CK7 is usually diffusely, strongly expressed in all or most salivary gland carcinomas [460], whereas it

is absent in OSCC with rare exceptions, although it can be seen in oropharyngeal tumors [461]. The solid variant of adenoid cystic carcinoma may mimic basaloid squamous cell carcinoma, but the biphasic pattern, if present even focally, and expression of basal cell/myoepithelial markers and CD117 usually resolve the dilemma. Fluorescence in situ hybridization may be used for detecting loci altered in certain tumors of salivary gland but not of squamous epithelial origin: *MAML2* in mucoepidermoid carcinoma [462], *EWSR1* in clear cell/myoepithelial tumors [459, 463], *PLAG1* in pleomorphic adenoma, and *MYB-NFIB* in adenoid cystic carcinoma [464, 465]. Salivary duct carcinoma (SDC) can also closely mimic squamous carcinoma usually in cytology specimens. The reverse can be true, and squamous carcinoma involving salivary duct may be misinterpreted as SDC [466]. Occasionally, however, SDC can show an extensive bona fide squamous/squamoid component that we have seen in a tumor with apocrine differentiation as described by others [605]. The term adenosquamous carcinoma was proposed for this rare occurrence [605], but this designation should be probably best reserved for tumors arising from the squamous epithelium, and in the presence of adenocarcinoma in situ of salivary glands, these tumors are best classified as salivary duct carcinoma with squamous differentiation.

Spindle Cell Carcinoma Versus Sarcoma. Fortunately, true mesenchymal neoplasms are extremely rare in oral cavity. Generally (and statistically) speaking, a spindle cell neoplasm in the head and neck mucosal surfaces including oral cavity is likely a squamous cell carcinoma until proven otherwise. Clinical information may be critical for the correct diagnosis, and a clinicopathologic approach has the best chance of success in solving this at times difficult differential diagnosis since spindle cell carcinoma can downregulate or lose most (or even all) epithelial markers and many sarcomas can focally or diffusely express various cytokeratins as previously discussed. Several clues that may help both the clinician and the pathologist can be found in the clinical context: previous radiation history may indicate a secondary sarcoma, such as osteosarcoma or angiosarcoma, usually distinguishable by morphology and/or immunoprofile; patient's young age would point toward a sarcoma since, despite the increase in OSCC in the younger patient group noted in recent decades, spindle cell subtype is extremely uncommon in this population.

Immunoprofile is very important in separating spindle cell carcinomas from other mimickers. A caveat is that cytokeratin expression was reported only in 25–60 % of SPSCC [19], nonspecific mesenchymal markers such as vimentin are virtually seen in all cases, and one or even two different specific myoid markers can be expressed in up to one-third of SPSCC [322]. Other markers including p63, p40 may help pointing to the correct diagnosis [322, 467]. Rhabdomyosarcoma and synovial sarcoma frequently express cytokeratins and therefore may pose significant challenges. Immunohistochemical studies for rhabdomyosarcoma and fluorescence in situ hybridization or molecular studies identifying the specific gene rearrangements for the second are essential when these differential considerations occur. Carcinoma in situ/high-grade dysplasia in the adjacent mucosa or a discernible squamous component (keratin pearls, intercellular bridges) definitively establishes the diagnosis of spindle cell squamous cell carcinoma.

As previously discussed, some other variants of squamous cell carcinoma such as adenoid (acantholytic) type, in addition to mimicking an adenocarcinoma, can be confused with epithelioid angiosarcoma. Immunohistochemistry is essential and extremely helpful in this distinction since squamous cancer does not express vascular markers.

Mucosal melanoma is notoriously protean with both epithelioid and spindle cell morphology, among many others, and may superficially mimic a poorly differentiated or spindle cell squamous cell carcinoma; the intraepithelial component, if present, is distinct and immunoprofile entirely different, easily resolving the issue.

Noninvasive squamous proliferations vs. early invasive carcinoma is one of the most difficult encounters in oral neoplastic pathology as well as a common problem in the referral and consultation practices [455]. In our experience, while this can be occasionally a very challenging distinction, it is very commonly associated with either extremely thick, keratotic lesions, superficial or inadequate area sampled (center of erosion/ulceration), or a combination of these factors. Lack of definitive pathologic criteria to establish stromal invasion at its earliest certainly plays a role, and this topic will be discussed in detail below in Sect. 5.3.5.3.

Odontogenic carcinomas are extremely rare tumors that occasionally can mimic squamous cell carcinoma. Radiologic localization as well as typical histologic features of specific tumor types (such as nuclear palisading, inverse polarity) are helpful, whereas immunohistochemical studies are not.

Metastasis Versus Primary. The vast majority of oral squamous carcinomas are diagnosed at their primary site, but this clinical question occurs primarily in the setting of extensive, multifocal oral dysplastic lesions and certain entities such as proliferative verrucous leukoplakia, or when locoregional recurrence is suspected in a patient with previous OSCC history. Second primary tumors, either synchronous or metachronous, occur in 10–20 % of HNSCC patients [2]. Presence of a preneoplastic lesion usually confirms the origin of the infiltrative tumors but sometimes deceptive colonization of the native epithelium by the metastatic tumor can be misleading. This could be a potentially extremely important clinical question (recurrence versus new primary), and unfortunately very few tests validated for clinical practice are available to the practitioner today. We have encountered patients with metachronous carcinoma with a different HPV profile. Unfortunately, as discussed, HPV-related tumors are frequent in oropharynx but rare in oral sites. Further progress in gene sequencing techniques and defining distinct genetic signature of tumors sharing the same phenotype (keratinizing squamous cell carcinoma) may provide a useful and affordable clinical way to allow distinction of primary, secondary, and metastatic tumors in the near future.

5.2.8.2 Oral Squamous Cell Carcinoma Versus Benign Diseases

The significance of misdiagnosing a benign lesion as malignant is evident. Most lesions discussed below are quite rare; however, some can be frequently found in resection specimens, and diagnostic errors may have devastating consequences. Clinician and pathologist awareness of these rare occurrences is essential. The

surgeon or dentist should question a pathologic diagnosis at odds with clinical-radiologic impression, as this author has learned throughout the years. Some of the conditions below require only knowledge of the biopsy site and morphologic characteristics (organ of Chievitz), whereas others require extensive workup and clinicopathologic correlation.

Odontogenic rests have the morphology and immunophenotype of squamous cells but are usually bland and can be easily distinguished in a resection specimen by the experienced observer. Problems usually occur in small or crushed specimens. The juxtaoral organ of Chievitz is a structure of unclear function found in the retromolar area [468], likely an embryologic rest, comprising both squamous epithelial nests in close proximity to neural fibers mimicking perineural invasion or an ameloblastic neoplasm. It can be differentiated by its lack of cytologic atypia or mitotic activity and typical localization. Even in resected mandibles for oral squamous cell carcinoma, misinterpreting this normal structure as perineural invasion may potentially lead to unnecessary therapy (Fig. 5.12).

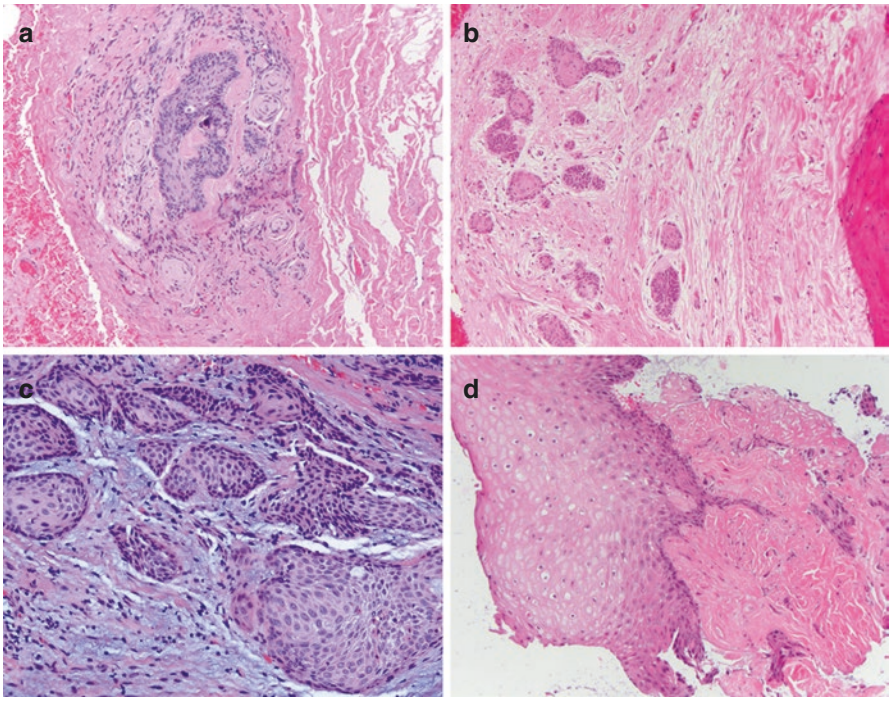

Fig. 5.12 Diagnostic pitfalls for oral cancer. (**a**) Organ of Chievitz, surrounded by neural fibers, may mimic carcinoma with perineural spread. (**b**) These odontogenic rests were seen in the maxillary bone resected for alveolar ridge cancer and may lead to upstaging if misinterpreted as invasive carcinoma. (**c**) Healing necrotizing sialometaplasia in a palate ulcer of a bulimic patient shows myxoid background simulating squamous cell carcinoma with desmoplastic reaction. (**d**) Radiation mucositis with extensive fibrosis and deep epithelial clusters adjacent to necrosis (*right lower*) mimicking invasive carcinoma

Pseudoepitheliomatous (pseudocarcinomatous) hyperplasia (PEH) (Fig. 5.13) is not a clinical or pathologic entity but simply a florid regenerative or reactive proliferation of squamous mucosa with an endophytic pattern of growth and no to minimal cytologic atypia, commonly encountered in oral cavity biopsy in a plethora of clinical circumstances. Similar changes are well described in skin [469]. Some degree of hyperplasia is almost always seen adjacent to a destructive, erosive/ulcerative deep lesion regardless of its underlying cause, and not uncommonly a downward growth would qualify the process as PEH. Because of its depth in the submucosa or even deeper structures and architectural (squamous pearls) and cytologic (increased mitoses, nucleoli, minimal nuclear anisocytosis) worrisome features, it can be mistaken for SCC. The reverse is rare but WDSCC often appears histologically extremely bland (even when clinically is an evident large neoplasm incompatible with any "hyperplasia") and mimics PEH in superficial samples resulting in diagnostic delays which could negatively affect outcome (Fig. 5.1). To compound the problem, PEH quite often accompanies other lesions such as necrotizing sialometaplasia (discussed below) and

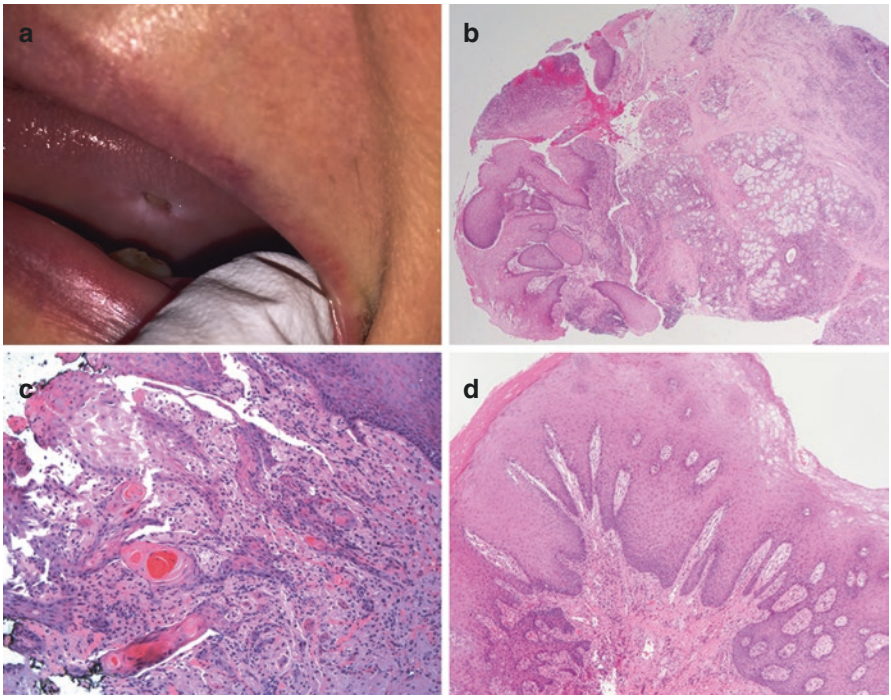

Fig. 5.13 Pseudoepitheliomatous hyperplasia (PEH). (**a**) This ulcer of lateral tongue showed PEH microscopically. Repeat biopsy confirmed its benign nature. (**b**) Epstein-Barr virus mucocutaneous ulceration can mimic cancer both clinically and histologically as seen in this immunosuppressed patient with extensive PEH (*left*) adjacent to the ulceration. (**c**) PEH mimics superficial SCC in a granular cell tumor of the tongue (*right lower*). (**d**) PEH (*right* and *center*) can coexist with dysplasia or carcinoma as seen in this hard palate lesion adjacent to microinvasive carcinoma (*left lower*)

is associated with florid reepithelialization/reparatory changes, both conditions with significant architectural and cytologic atypia and SCC mimickers on their own. Given that any erosive or ulcerative process is likely to be associated with a degree of hyperplasia, it is very likely that in fact only the more severe changes are noted and PEH is probably underreported both in the literature and in pathology reports.

Several underlying causes of PEH are well described including tumoral, infectious and inflammatory and iatrogenic: Both benign submucosal (granular cell tumor) and malignant (lymphoma, melanoma [470, 471]) neoplasms are associated with florid PEH. The most commonly described and probably over-referenced neoplastic-related PEH is supradjacent to granular cell tumor, a very rare soft tissue neoplasm (Fig. 5.13). Oral submucous fibrosis, a preneoplastic lesion that will be discussed later, can be also seen adjacent to PEH [472].

Infectious and Inflammatory Conditions. Numerous deep fungal infections are associated with PEH: *Histoplasma*- [473] and *Candida*-associated squamous proliferations such as median rhomboid glossitis and hyperplastic candidiasis [474], but also virally induced lesions, including the recently described immunosuppression-associated EBV-related mucocutaneous ulcerations (Fig. 5.13) [475], may closely mimic both clinically and pathologically squamous cell carcinoma. Other immune-mediated conditions such as lupus erythematosus or lichen planus lesions, either hyperplastic or erosive, often can be associated with PEH as well.

Iatrogenic (surgical, radiation or chemotherapy) mucosal injuries and regenerative changes leading to scarring and fistula may exhibit prominent PEH. In the setting of a large referral or cancer center setting, the most common clinical scenario is that of a lesion-developed post-therapeutically adjacent to, or at some distance from, the surgical site. It may pose significant diagnostic difficulties since most of the patients underwent therapy for squamous cell carcinoma, the leading differential consideration. Postsurgical alterations [476], palatal papillary hyperplasia, dental prosthesis -induced injury, jaw osteonecrosis [477], and essentially any traumatic mucosal lesion may be accompanied by PEH. Although a recent report in the animal model described PEH more commonly occurring after laser therapy compared to scalpel surgery-induced wounds [478], it is certainly not limited to that type of procedure.

While misdiagnosing PEH as well-differentiated squamous cell carcinoma is by far the primary concern and most likely error, we have encountered rare occasions where recurrent, fistula-forming SCC was repeatedly misdiagnosed as PEH in biopsy samples (Fig. 5.14) due to its bland appearance, a pitfall described in cutaneous tumors [479]. Of course, PEH can be seen adjacent to carcinoma (Fig. 5.13).

Immunohistochemistry may be valuable in this differential diagnosis since SCC overexpresses p53, exhibits higher ki67 proliferation index, and losses e-cadherin at the invasive front [480, 481], but an overlap exists and the immunoprofile may be misleading. Recently, a molecular approach discriminating between SCC and PEH was reported in cutaneous tumors, but has yet to be confirmed in the mucosal sites [482]. Careful histopathologic evaluation, knowledge of the clinical setting, and close communication between the clinician and pathologist are key and remain the mainstay in preventing diagnostic errors. Rebiopsy with ample sampling of the interface may be required (Fig. 5.1).

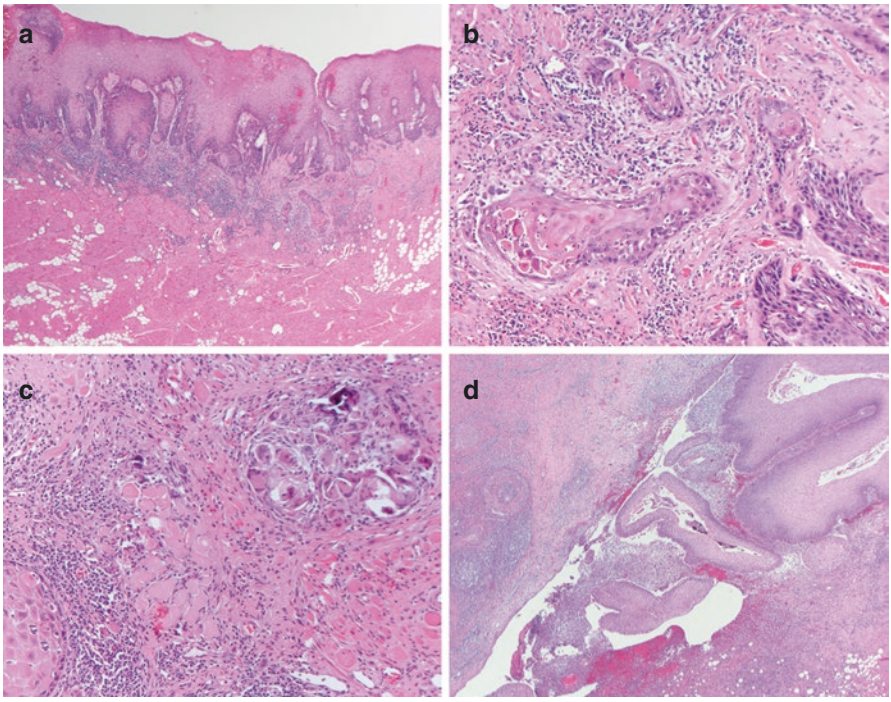

Fig. 5.14 Squamous carcinoma inflammatory response. (**a**) Tumor lymphoid response is often a harbinger of early microinvasion as seen in this erythroplakia where it is prominent adjacent to the invasive (*left half*) but not to the dysplastic (*right*) component. (**b**) Any keratinizing carcinoma may elicit an acute neutrophilic response often causing acantholysis and microabscess as seen in this early oral tongue cancer. Presence of acute inflammation and granuloma may be misinterpreted as reactive. (**c**) Keratin extruded from neoplastic cells often causes an exuberant foreign body giant cell reaction with microcalcifications (*right upper*). Tumor cells may be away as seen in this oral tongue cancer (*left lower*) or even absent in small biopsies. Foreign body granuloma is a suspicious finding in oral mucosa, and rebiopsy should be considered in these cases (**d**) Some tumors can mimic clinically and histologically a fistula tract, particularly recurrences in the radiated field, such as this buccal cancer where several biopsies were required for positive diagnosis and earlier samples interpreted as abscess and granulomatous inflammation

Inclusion cysts are common in skin but rare in oral cavity. A common occurrence in mucosal OSCC is a foreign body giant cell reaction to the keratin produced by tumor. This can be quite extensive at times obscuring the neoplastic cells (Fig. 5.14). Presence of keratin in foreign body giant cells is highly suspicious for an adjacent carcinoma, and both the pathologist and the clinician should consider additional sampling when this occurs (levels and repeat biopsy, respectively). These findings may be extensive in fistula-forming OSCC and in postradiated tumors.

Necrotizing sialometaplasia (NSM) (Fig. 5.12) is a self-limiting condition described in 1973 [483] and characterized clinically by mucosal ulceration and histologically by pseudoepitheliomatous hyperplasia, salivary gland lobular necrosis, and exuberant squamous metaplasia of the ducts [483, 484]. It heals in

2–8 weeks without treatment. Often its cause is unknown and historically an ischemic mechanism was postulated; however, similar to PEH, it may be seen in a variety of conditions, including after surgical or radiation therapy. Recently, reports of NSM associated with eating disorders, where the causative injury is likely repetitive mechanical trauma, are increasing [485, 486]. NSM may initially appear as a nodule which rapidly progresses to a crater like ulceration [486] or presents as an ulcer involving most commonly the palate minor salivary glands.

NSM may closely mimic squamous cell or mucoepidermoid carcinoma due to its depth and atypical distribution of squamous nests and occasional presence of mucus pools and mucocytes (Fig. 5.12). Clues for the examining clinician are the rapid development and history of radiation mucositis, local injury, or eating disorder [486]. Main clues for the pathologist are preserved lobular architecture and cell composition, incongruent with mucoepidermoid carcinoma, and residual myoepithelial cells around some, if not all, ductal structures involved, best highlighted by ancillary studies which may be helpful in selected cases [487, 488].

Radiation-induced changes pose one of the most difficult histologic differential diagnoses in head and neck and oral pathology. The cytologic atypia can be quite dramatic and may involve the epithelial lining, submucosal minor salivary gland, endothelial cells, or stromal mesenchymal cells [489]. The epithelial atypia and the proliferation index are very similar to that seen in oral dysplasia [490], and attempting to diagnose low-grade dysplasia in irradiated mucosa is probably not wise. As previously discussed, presence of both PEH and NSM is very common and compounds the issue since irradiated proliferative regenerative squamous mucosa may be virtually indistinguishable from residual or recurrent squamous cell carcinoma (Fig. 5.12). Immunohistochemistry for epithelial and myoepithelial markers may be helpful in the differential with the caveat that aberrant expression and increased proliferation can be seen in reactive conditions [490].

Benign/reactive spindle cell proliferations include granulation tissue, inflammatory pseudotumor, as well as low-grade neoplasms such as inflammatory myofibroblastic tumors. The morphologic findings can be misleading due to nuclear atypia, high mitotic rate, and myxoid changes mimicking desmoplastic reaction. Differential diagnosis from spindle cell carcinoma was discussed under the respective section [467].

Osteonecrosis. An excellent example where discrepancy between an innocuous clinical exam and concerning histopathologic features should help the team reach the correct diagnosis is that of bone pseudoinvasion associated with jaw osteonecrosis. Often, the necrotic bone curetted in the clinic and adjacent soft tissue are not sent for histologic evaluation, and therefore general, head and neck, and oral pathologists may be less familiar with this finding. A combination of pseudoepitheliomatous hyperplasia juxtaposed to, and occasionally wrapping around the, necrotic bone might raise the question of an invasive process [63, 606]. Pseudoinvasion of bone in osteomyelitis resulting from mucosal ulcers or fistula tract has been previously reported [491], but due to the increasing prevalence of

osteonecrosis induced by bisphosphonate seen in clinical practice, we and others remarked an increase of these findings in the last decade. Pseudoepitheliomatous hyperplasia has been previously described in both bisphosphonate and radiation-induced osteonecrosis [477, 606] and usually lacks significant atypia [63]; however, we had seen occasional cases with significant cytologic atypia that appears to invade the necrotic bone [606], likely related to either radiation effect or reepithelialization at the mucosal defect site. None of these cases showed a desmoplastic reaction which may be valuable discriminating finding from residual squamous cell carcinoma that may be present in the irradiated field with osteonecrosis. Unlike true bone invasion, the squamous epithelium is in tight contact with the necrotic bone [491, 606]. In cases of bisphosphonate-associated osteonecrosis, some atypia may still be present but is reactive in nature. The underlying disease is most likely breast carcinoma or multiple myeloma [477, 606] unlike osteoradionecrosis which is a consequence of radiotherapy for head and neck cancer, and the pathologists would benefit from this key clinical information in their assessment (Fig. 5.9).

5.3 Potentially Malignant Disorders and Early Oral Cancer

5.3.1 Definition

Potentially malignant disorders (PMDs) are defined as oral disorders that *may* progress to oral cancer. PMD designation has been recently proposed following a WHO Working Group consensus meeting [492] to encompass both premalignant lesions (*localized* oral alterations) and premalignant conditions (oral manifestation of a *systemic* disorder) acknowledging that distinction is not always clear between these two categories previously endorsed in the earlier WHO classification. PMDs include a variety of heterogeneous conditions, of which the most common is leukoplakia, with a variable risk of malignant progression. Excluding known conditions without risk of neoplastic transformation is a diagnostic requirement of PMD [20, 492]. Ideally, PMD risk of transformation or progression to cancer should be assessed by joint clinical and histologic evaluation and an overall risk stratification applied. Such system does not exist to date, and although in general there is good macroscopic-histologic correlation (clinical high-risk lesions are associated with high-grade dysplasia), discrepancies between the clinical and histologic findings exist, and there is a stringent need for identifying, validating, and eventually integrating biomarkers in the risk stratification schema.

5.3.2 Synonyms

Precancerous (or premalignant) conditions and lesions, epithelial precursor lesions [502], squamous intraepithelial neoplasia.

5.3.3 Epidemiology

PMD prevalence is estimated at 2.6 % worldwide [493, 494], but it varies significantly based on the study design, geographic region, country, and patient population, and the reported range is much broader, from 0.2 to 11.3 % [495]. Oral cancer incidence is inversely related to the socioeconomic status and is the sixth most common cancer worldwide but the most frequent cancer in South and Southeast Asia [13]. Prevalence of PMD parallels the oral cancer statistics [13], presenting both an opportunity and a challenge to identify high-risk patients early and address the premalignant or early malignant lesion at its inception.

5.3.4 Clinical and Macroscopic Aspects

Leukoplakia is defined as "white plaques of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer" [492]. The term leukoplakia carries exclusively a clinical connotation and should not be used by the pathologist. This diagnosis requires the exclusion of several white lesions demanding astute clinical diagnostic skills and knowledge of various conditions including white sponge nevus, frictional keratosis, traumatic (morsicatio) or chemical injury, infectious diseases (acute pseudomembranous candidiasis, hairy leukoplakia), leukoedema, nicotine stomatitis, but also histologic exclusion of other nonneoplastic disorders (such as lichen planus and discoid lupus). A biopsy, with or without ancillary studies if infectious agents are suspected, is considered mandatory after a provisional diagnosis of leukoplakia was established [492]. Leukoplakia occurs typically in the smoker man in the fifth to seventh decade with an estimated 1–5 % worldwide incidence (variable with region, underlying etiology, epidemiologic and genetic factors) [494, 496].

Sites of involvement vary with underlying etiology, and buccal-gingival sites are more common where chewing tobacco, betel, or areca nut is prevalent, whereas smoking-related leukoplakias involve the oral tongue and floor of mouth [20, 23, 494]. The anatomical subsite is important since it appears to be an inverse relationship between their prevalence and risk of transformation [20, 23] and should be clearly indicated in the clinical note at the time of evaluation. Thorough documentation of its appearance and size is essential for surveillance and clinicopathologic assessment.

A white patch that cannot be removed by scraping, fulfilling the exclusionary criteria above, has been historically classified based on its appearance into two main categories: homogeneous and nonhomogeneous leukoplakia. The distinction between the two is somewhat imprecise as it was pointed out [20, 492] but remains important given the different biologic significance. *Homogeneous* leukoplakia is uniform in appearance, usually thin (but it can be thicker), and leathery with occasional surface superficial cracks. Several types of *nonhomogeneous* leukoplakias are described: (a) s*peckled* (or mixed, predominantly white patch with admixed red areas, essentially a form of *erytroleukoplakia*, and, in this author opinion, better

classified under this latter category); (b) *nodular* (granular) with polypoid excrescences, or small nodules in an otherwise flat plaque; and (c) *verrucous* leukoplakia which is defined by its wrinkled, cauliflower-like, white or gray appearance while preserving a flat shape (not tumor forming). It may be solitary or multifocal; in the latter case, considering proliferative verrucous leukoplakia, a distinct clinicopathologic entity, is important. Several studies have convincingly shown that nonhomogeneous leukoplakias have a higher risk of transformation compared to the homogeneous types [497–500]. A uniform classification and staging system of leukoplakia was proposed [501], but it has not been widely accepted or employed in clinical practice. The recent consensus approach to PMD diagnosis and classification may fuel some optimism for nomenclature standardization in the future, indispensable for any progress in the field [492, 502].

Oral leukoplakia is the most common PMD, having an estimated average prevalence of 2 % worldwide [493, 494] and carries a definite risk of progression to oral cancer, risk difficult to quantify and widely variable in the literature based on the study design, region, economic status, and population included. Most leukoplakias do not progress and can regress either spontaneously or after smoking cessation. Its average *annual* malignant transformation rate is estimated at 1 % [503]. The overall leukoplakia transformation rate is estimated at 5 %, but rates between 0.13 and 36.4 % are reported in the literature [504, 505]. Several intrinsic or extrinsic factors may increase or decrease leukoplakia malignant potential as will be discussed. Wide variation in study design, the methods for calculating the transformation rate regardless of time for follow-up as recently pointed out [506], major expected differences between general- and hospital-based populations [494], and inclusion criteria-all significantly hamper comparison of various studies results. For every clinical significant factor/identified to date by several groups, conflicting data are readily found in the literature, to the dismay of the lucid reviewer [505].

The least frequent clinical characteristics are generally associated with higher-risk leukoplakia: women were shown to have a higher risk of progression than men at least in Western studies [494, 498, 507]; lesions in nonsmokers (idiopathic leukoplakia) have higher propensity for transformation than smokers [498, 507, 508]; areas of higher risk (floor of mouth, ventrolateral tongue) are the most uncommon sites for leukoplakia but twice more likely to transform [494] than the lesions in lower risk, high prevalence subsites such as buccal and gingival mucosa [23, 509]; nonhomogeneous leukoplakia was shown to carry a seven times relative risk of progression to cancer than homogeneous leukoplakia [499]; and about 5 % of homogenous leukoplakias are expected to develop cancer [494]. Overall, half of leukoplakias will persist, up to 40 % will regress, and only a minority will increase in size, develop new lesions, or progress to invasive carcinoma [494].

Size of the plaque was found to be an independent prognostic factor by several groups: the larger the lesion, the higher the risk [494, 499, 508, 510]; however, it should be remembered that carcinoma can be seen in small lesions (Figs. 5.1 and 5.2). Time for transformation varies, but in average it was reported around 33 months in two studies [509, 511], and most leukoplakias that will progress will do so in the first 5 years with the transformation rate decreasing thereafter [494]. The presence

of dysplasia in leukoplakia is the single most significant predictor as shown in multiple studies [494, 498, 505, 506, 512].

Histopathologically, leukoplakia is invariably characterized by hyperplastic squamous epithelium, with or without dysplasia. The white color seen at clinical examination is due to superficial keratin production: hyperkeratosis (anucleated keratin in the corneal layer), parakeratosis (nucleated keratinocytes), or a mixture of the two is always seen in white patches (Figs. 5.15, 5.16, and 5.17). The difficulty in diagnosis occurs in more proliferative lesions with verrucoid or downward pattern of growth. In the latter, separating the earliest point of invasion may be extremely difficult particularly in thick, large lesions. Given their intrinsic heterogeneity, sampling error is another documented potential cause for diagnostic error, and therefore, biopsying multiple areas of large lesions should be considered in order to mitigate this risk [36].

Erythroplakia is defined as a "fiery red patch that cannot be characterized clinically or pathologically as any other definable disease" for the last four decades [513], and this definition was preserved in subsequent consensus meetings [492, 519]. It is a condition seen in elderly adults (sixth to seventh decade) in the Western world but occurs in younger patients in India and has a strong association with alcohol and tobacco consumption, either smoking or chewing [515]. While it is a much rare condition with a worldwide prevalence of less than 1 % [516], it commonly shows high-grade dysplasia [502] and has the highest risk of transformation to carcinoma from all oral premalignant disorders [102, 498, 516]. The most commonly involved subsite is soft palate, and early biopsy is recommended and mandatory for diagnosis. Clinical examination and histopathologic evaluation are required to exclude various mimickers, including but not limited to erosive disorders, desquamative gingivitis, discoid lupus, erosive lichen planus, pemphigoid, erythematous candidiasis, and other inflammatory/infectious conditions. This misclassification may result in aggressive management and unnecessary procedures, as we have occasionally encountered in our practice (Fig. 5.20).

While pure erythroplakia is relatively rare, *speckled erythroplakia* is one of the most frequent nonhomogeneous variety of leukoplakia [492], and its potential for transformation falls between that of leukoplakia and erythroplakia. Because of the classification problems, overlap between the red, white, and mixed lesions, reviewing the literature on the subject and comparing divergent results is difficult [505, 516], and the confusion between speckled leukoplakia and speckled (granular) erythroplakia (based on the relative predominance of the red or the white color of plaque) [516] prompted some authors to eliminate any mixed lesions from their analysis [102]. Most investigators, however, have retained the mixed lesions in their studies. For example, cases of "leukoplakia erosiva" [497] of which 26 % progressed to squamous cancer probably belonged to the erythroleukoplakia category. A uniform terminology and clinical designation of these lesions is long overdue.

Histologically, erythroplakia is often associated with high-grade dysplasia, carcinoma in situ, or invasive carcinoma in over 90 % of cases even at the time of the diagnosis [102], and therefore it is considered a high-risk lesion regardless of the

involved subsite. The red color in this author experience is related to stromal involvement changes, including microscopic surface epithelial erosion and submucosal hemorrhage (Fig. 5.2). Unlike leukoplakia, homogeneous erythroplakia lacks parakeratosis and even hyperplasia, and mucosa can be thin or even atrophic. Focal erosion may be detected at clinical level as well.

Leukoerythroplakia, as already alluded, is usually classified either under nonhomogeneous leukoplakia or, if red areas predominant, under erythroplakia. Given this diagnostic and nosologic uncertainty, it is difficult to assess its epidemiology or specific histopathology, if any. For practical purposes, any mixed lesions should be considered high risk and biopsy targeted to the red areas.

Proliferative verrucous leukoplakia (PVL) is currently considered a potential clinicopathologic entity and not simply a variant of leukoplakia. Since it was described three decades ago [517], several small studies and numerous case reports have been published, but, as pointed out recently, its etiology and clinical and

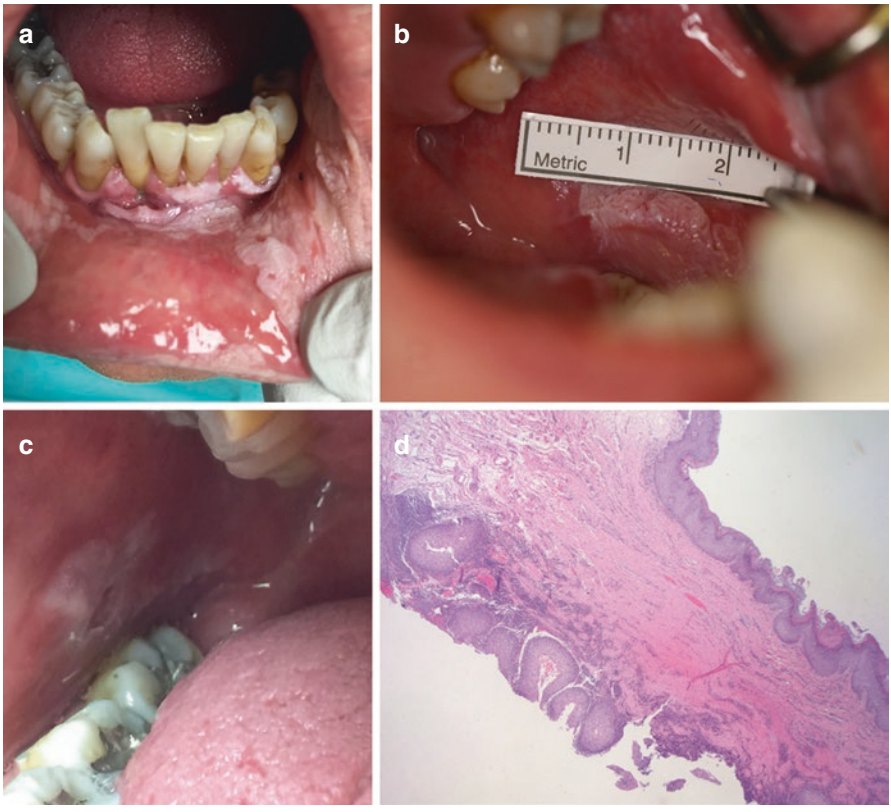

Fig. 5.15 Proliferative verrucous leukoplakia in a patient with 9-year history of multifocal *white* plaques of gingivolabial (**a**) and bilateral buccal (**b, c**) sites. Most lesions are verrucoid (**a**, **b**), but some are flat (**c**). (**d**). Gingival lesion biopsy showed verrucoid hyperplasia (*right*) and early carcinoma (*left upper*)

histologic diagnostic criteria remain elusive [518]. The most recently proposed diagnostic criteria (not yet widely accepted) are mainly clinical, including presence of more than two oral subsites; aggregate size ≥3 cm; at least 5 years evolution with enlargement of existing lesions and occurrence of new ones; and biopsy exclusion of verrucous or squamous cell carcinoma [519], schema representing an update from an earlier proposal [520]. PVL is defined by multifocal, progressive leukoplakia that increases in number, size, and thickness progressing from flat to verrucoid, exophytic plaques and, almost inevitably, to oral cancer in most patients over a variable period of time.

PVL occurs predominantly in women with a median age over 60, and no association was found with smoking or alcohol, only about a third of patients being smokers [517, 521, 528]. Despite earlier claims [607], recent studies could not confirm a viral association with this condition when testing for human papillomavirus and Epstein-Barr virus was carried on with rigorous methods [522–524]. PVL affects primarily the buccal mucosa, tongue, and gingiva/alveolar ridge. Diagnosing early PVL is challenging, if not impossible [525], given that presumably the disorder commences with a single lesion when the distinction for trivial leukoplakia is not feasible. Only lesion(s) temporal progression in size, quality (flat to verrucoid), and number will reveal the diagnosis. Of note, the term proliferative is not related to the lesions' cell mitotic index but to their propensity to increase in number and to promptly recur after removal [505]. Progression to cancer occurs after a variable interval, in average 7.7 years [528], in about 70 % of the patients (range 60–100 %) [526], but we have seen new cancers occurring up to two decades after initial presentation, with multiple lesions undergoing malignant transformation metachronously. Life-long follow-up is therefore essential although optimal management is controversial [527]. Gingival-alveolar PVLs were reported to have a higher risk for transformation by some [528–530], but any site is at risk. A recent study suggests that oral cancer arising in PVL may be more indolent than conventional oral carcinoma [531] which is not surprising given the prevalence of verrucous or hybrid carcinomas in PVL patients, tumors with less aggressive biology than conventional OSCC. While many reviews and several studies discuss transformation, the data on cancer-specific survival of PVL-related cancer is relatively scant, due to small cohort size and relative short follow-up period for this protracting disease in most studies.

Clinical differential diagnosis in a solitary early lesion includes usual or verrucoid leukoplakia, particularly at the early stages, and, later in the disease, multifocal leukoplakia, not PVL-type, distinction that can pose both clinical [532] and histologic classification challenges [518]. Because it is now recognized that not all PVL lesions are clinically verrucoid (Fig. 5.15), the alternative designation of proliferative *multifocal* leukoplakia was recently suggested [533]. (The reverse is true; there are verrucoid lesions that are not PVL.) The temporal element is another difficult clinical proposition: lesion(s) persistence for at least 5 years was a minor criterion in the early diagnostic proposal [520] and became a requirement in an alternative, contracted form of the initial proposal [519].

Histologic findings are nonspecific, but various degrees of hyperplasia, either flat or verrucoid, associated with hyper(para)keratosis are the presenting microscopic features in over half of the cases [528]; carcinoma was reported at presentation in 10 of 54 patients in the same study. As previously discussed under verrucous carcinoma, verrucous hyperplasia is a relatively contested entity introduced in 1980 [534] which some believe to represent early PVL in many cases [527] and others view it as an early verrucous carcinoma [293, 525, 535]. There were ten histologic stages proposed in the original PVL description [517], subsequently reduced to four by others who preserved only flat leukoplakias without dysplasia, verrucous hyperplasia, verrucous carcinoma, and squamous cell carcinoma [266, 293]. A more practical approach would be to collapse the histologic spectrum in two main categories: noninvasive and invasive lesions. The invasive component should be clearly described as conventional squamous cell or verrucous carcinoma. Even this dichotomy may be difficult to ascertain in small biopsies or early infiltrative tumors. Excision or some form of treatment may be advisable for all verrucoid leukoplakias, but in the typical PVL patient, with multifocal such lesions, risk stratification and prioritizing treatment may be the main clinical concern.

Regardless of how many histologic mileposts are considered in this disease continuum, given that most of these lesions show only low-grade keratinizing dysplasia, histologic predictive power of a biopsy sample is uncertain at least at early stages. Later in the course of the disease, development of increasing grades of dysplasia in these hyperplastic lesions may precede eventual progression to well-differentiated keratinizing squamous cell carcinoma, verrucous carcinoma, or so-called hybrid forms with mixed histologic features. Papillary carcinoma is likely not seen in PVL patients [535] despite the original description. Differential diagnosis includes other types of oral keratoses, verrucous hyperplasia, and lichen planus, the latter due to the fact that many keratotic dysplastic lesions are associated with a dense lymphoid infiltrate [526, 528] (so-called lichenoid dysplasia). Distinguishing early verrucous carcinoma from a thick verrucous hyperplasia may be extremely challenging even in excision specimens, let aside in a biopsy, since they are primarily separated by the endophytic and exophytic growth, respectively and depth of involvement [266]. VC can certainly have at the least an exophytic component in rare occasions [18], and dysplastic viral papillomas can enter the differential diagnosis in these cases. In fact, distinguishing various exophytic-papillary and verrucoid oral lesions is a common diagnostic dilemma in clinical practice, and some gray zone areas exist not only between verrucoid hyperplasia, verrucoid carcinoma, and PVL but also between verrucoid and papillary lesions [536]. Small bioptic samples may require an indeterminate classification, and final diagnosis is often deferred for excisional sample evaluation of the entire lesion.

In sum, as pointed out, lack of uniform clinical criteria for PVL diagnosis and macroscopic and histologic overlap with other lesions and nonspecific histologic changes make the comparison of published data extremely challenging [503, 526]. PVL remains a difficult to diagnose disease especially at an early stage. Any verrucoid leukoplakia or presence of multiple white plaques should alert the clinician at

the possibility of this rare disease, whereas presence of verrucoid hyperplasia is a clue for the pathologist. Given its well-documented propensity for progression to carcinoma, this team effort is worthwhile for early diagnosis, for which clinicopathologic communication and ample sampling of lesion(s) offer best chance. To date, there are few entities for which the need for consensus and uniform definition is more stringent for developing evidence-based diagnosis and management guidelines.

Oral submucous fibrosis is a well-described and universally accepted potentially malignant disorder associated with chewing areca nut product (betel quid and gutkha being most commonly used) [492, 537]. Oral submucous fibrosis and its preneoplastic potential were recognized for five decades [538]. It is characterized by extensive subepithelial fibrosis involving the oral cavity, oropharynx, and proximal esophagus. There are four consecutive histologic stages of the diseaseat: at presentation, edema and fibroblastic proliferation and acute inflammatory infiltrate; followed by increased thickened collagen fibers and diminished fibroblastic and inflammatory response; followed by more extensive hyalinization with chronic inflammation; and, in final stage, lamina propria is diffusely replaced by hyalinization with epithelial atrophy [525]. Characteristically, squamous dysplasia is not prominent and is missing in most cases of OSF, occurring only in 7–26 % lesions. The clinician should not be reassured since 7–13 % of OSF will progress to squamous cell carcinoma [539]. OSF-associated SCC cannot be morphologically distinguished from conventional tumors. Clinical diagnosis of the condition is key [540] and the role of histopathologic examination in the management of the disease is currently unclear [541], but evaluating the overlying mucosa to identify and grade dysplasia is recommended.

Oral lichen planus (OLP) is a chronic immune disorder of unknown etiology defined by increased subepithelial T lymphocytes, hyperkeratosis, and erythema with possible erosion or ulceration [492]. Whether OLP is a preneoplastic lesion has been long debated [492]. Widely variable diagnostic criteria used in clinical practice likely play an important role in conflicting results and divergent opinions fueling the controversy. If *all* the clinical criteria (bilateral, symmetric lesions with reticular pattern; erosive, atrophic, bullous, and plaque-type lesions accepted only when reticular lesions are present elsewhere) and histopathologic criteria (well-defined, band-like, mainly lymphocytic infiltrate confined to the superficial connective tissue; liquefaction degeneration of the basal layer; absence of epithelial dysplasia) are fulfilled, then a diagnosis of oral lichen planus can be made. If most but not all of either clinical or pathologic criteria are met, then the term oral lichenoid lesion (OLL) is employed [542].

The main criticism and crux of the long-standing controversy is whether presence of dysplasia is acceptable for the OLP diagnosis in a patient that otherwise meets the other criteria. This is a somewhat circular argument, since obviously eliminating dysplastic lesions may bias the selection of an OLP subset that truly has the potential to progress [543]. Most oral pathologists are familiar with the rich "lichenoid" inflammatory infiltrate, usually admixed with numerous plasma cells (uncommon in OLP) and associated with various dysplastic keratoses, particularly early cancer (Figs. 5.2, 5.16, and 5.17) or high-grade keratinizing

dysplasia (Figs. 5.16, 5.17, and 5.20), where lichen planus is not a clinical consideration, and therefore avoid using "lichenoid dysplasia" designation alltogether [544].

Prospective studies from one group using dysplasia as an exclusion criterion from the OLP group [542, 545] found that oral lichen planus had negligible malignant potential, whereas the oral lichenoid lesions were most likely to transform. This was confirmed by a recent rigorous literature review [543] which reported 1.09 % and 3.2 % malignant transformation rates of OLP and OLL, respectively. At particularly high risk for transformation are graft-versus-host disease-related oral lichenoid lesions, particularly when erosion is present [543, 546, 547].

The histopathologic features may be important in distinguishing the inflamed dysplastic mucosae from true lichen planus-induced basal atypia, another main contention point between various studies and researchers. Any interface inflammation is expected to induce cytologic alterations in the basal layer, yielding some degree of epithelial atypia [544]. Some authors suggested that lichenoid dysplasia represents a distinct form of leukoplakia resembling lichen planus clinically and histologically [546]. As previously stated, PVL in early stage can share clinical and histologic features with OLP, distinction extremely important for the significant difference in outcome and malignant potential of the two conditions [544].

Other preneoplastic conditions include actinic keratosis (see discussion in the lip cancer section), palate lesions in reverse smokers, discoid lupus erythematosus – controversial, particularly on the lip – and genetically inherited disorders including, but not limited to dyskeratosis congenita [492].

5.3.5 Microscopic Aspects of Potentially Malignant Disorders

After evaluating the relative risk of various clinical and macroscopic parameters described above, histologic evaluation assessment should be performed in all or most strictly defined potential malignant disorders [492]. The biopsy is deemed mandatory, and choosing the area to be biopsied, the sampling quantity and technique employed are all essential in obtaining a representative sample of the lesion. Due to these lesions heterogeneity, site and size of the sample are critical in providing the most representative or worst area of the lesion; two or more biopsies should be considered in large or multiple lesions [36].

Leukoplakia is not a pathologic diagnosis, but all these lesions show increased superficial keratin, either parakeratosis or hyperkeratosis, with or without dysplasia. Generally speaking, the risk of cancer progression is small (but not nil) in homogenous leukoplakia without dysplasia and increases with the degree of dysplasia [509, 549–552], with only a minority of studies not corroborating the traditional theory [36, 553]. The presence and grade of these preneoplastic alterations are the most important predictors of malignant progression. In a meta-analysis of 14 studies, 12 % of the dysplastic PMD progressed to carcinoma (range, 8–18 %) over an average period of 4.3 years [549].

Understanding the concept of oral epithelial dysplasia (OED), its predictive value, terminology variability, and practical application limitations are all important for the treating physician. Dysplasia is an artificial concept representing the phenotypic projection of a continuum of progressive genetic alterations with increasing likelihood for cancer transformation. The diagnosis of dysplasia is based on criteria that are overall well accepted; however, their relative weight is not specified and varies widely from one observer to the next (Table 5.3). While in other squamous mucosa such as the cervical mucosa, the cytologic criteria are preeminent, in oral cavity the architectural disarray may be more important as it will be discussed. There is currently no international worldwide consensus on which system to be used, obviously a major impediment to comparing the data and uniformly apply research findings into clinical practice. WHO suggests a five-tier system [502] while acknowledging alternative classification systems, reviewed extensively elsewhere [525, 554]. Unfortunately, the overlap between various categories in these different systems is far from ideal. Older systems are cumbersome, while others validated in the larynx have questionable applicability to oral mucosa [555, 556]. We will limit the discussion below to the WHO categories, analyze the underlying roots of its limitations, describe additional histologic criteria, and review recent proposals for alternative classification systems with putative clinical utility.

WHO classification of dysplasia foundation is the classical three-tier division (mild, moderate, and severe) of the mucosal epithelial surface, with two additional categories at the ends of the dysplastic-neoplastic spectrum (hyperplasia and carcinoma in situ, respectively). The consideration primarily of architectural features and only secondary of cytologic atypia, as described in a recent consensus review [554], reflects this author's experience. Moreover, the statement that "magnitude of surface keratinization is of no importance in the assessment of dysplasia" [554], crucial in oral dysplasia, is entirely accurate and widely recognized by oral and head and neck pathologists. However, these oral mucosa-specific features of dysplasia, already mentioned in practice guidelines [11, 12], are probably not universally accepted or applied by most general and community pathologists.

Table 5.3 World Health Organization criteria for diagnosing oral dysplasia [502]

Architecture	Cytology
Irregular epithelial stratification	Abnormal variation in nuclear size (anisonucleosis)
Loss of polarity of basal cells	Abnormal variation in nuclear shape (nuclear pleomorphism)
Drop-shaped rete ridges	Abnormal variation in cell size (anisocytosis)
Increased number of mitotic figures	Abnormal variation in cell shape (cellular pleomorphism)
Abnormally superficial mitoses	Increased nuclear-cytoplasmic ratio
Premature keratinization in single cells (dyskeratosis)	Increased nuclear size
Keratin pearls within rete pegs	Atypical mitotic figures
	Increased number and size of nucleoli
	Hyperchromasia

One vaguely defined concept is that of "keratinizing dysplasia" which is perhaps one of the most important causes of variation in oral dysplasia grading and diminishes its predictive power for preneoplastic lesions with extensive keratinization. Keratinizing dysplasia is an elusive concept, currently poorly defined although present in most textbooks [1, 557] but not in the WHO monograph [502]. It has been proposed under the term squamous intraepithelial neoplasia (SIN) [560], which is currently listed as an alternative classification in the WHO monograph [502]. We found these changes quite often adjacent to many small oral cancers showing minimal cytologic atypia but more significant architectural disarray. Keratinizing dysplasia may be the most common histologic lesion precursor to oral cancer. Unfortunately, a uniform system of grading keratinizing dysplasia does not exist, since it currently lacks even a generally accepted definition; other than that it is recognized as a precursor to oral cancer which does not require full mucosal thickness involvement to progress to carcinoma (Fig. 5.17). Of note, there is currently no definition or widely accepted spectrum for *nonkeratinizing* dysplasia; therefore, the separation of the two is extremely unclear. This nosologic confusion leads to a variety of designations in the literature such as "differentiated dysplasia," another gynecologic pathology import from the vulvar preneoplastic terminology [558], recently used by some authors for dysplasias found adjacent to 41 % (63/155) of oral cancer [559]. It is common for oral small well-differentiated squamous cell carcinoma to arise from mucosa with minimal, if any, atypia, thus keratinizing (or "differentiated") dysplasia. The concept of progressive dysplasia involving all three epithelial layers, with a purportedly increasing risk for transformation, is central to current WHO classification but is also imported from uterine cervix (cervical intraepithelial neoplasia, CIN 1–3), quite distinct from an embryologic, histomorphologic, and etiopathologic perspective from oral mucosa preneoplastic process, distinction recognized for a long time by both head and neck and oral pathologists [560, 561].

As a general rule, dysplastic lesions with flat base usually carry the lowest risk for transformation, whereas proliferative lesions with endophytic, spiky, irregularly fused, with abrupt acute angle subdivision, or with horizontal subepithelial growth are the most concerning for a high-risk lesion or even early invasion. This observer draws an imaginary line at the top of rete ridges and assesses the cytological and architectural atypia in the area *below* that line to assess both cytologic atypia and loss of polarity. That imaginary line is often drawn superficially to only the lower third layer if a strict topographic assessment is applied. Underdiagnosis of histologic alterations as no- or low-risk dysplasia is not uncommonly detected at retrospective review of the original biopsies taken at the same mucosal site where patients subsequently develop oral cancer, some months or years later and our experience was reportedly shared by others [562].

Uniform usage of the same terminology and classification system is important not only in comparing the data from various studies but also to set up a validated system for clinical practice and future research in identifying new biomarkers to objectively segregate the PMD intrinsic progression risk.

Squamous hyperplasia (or acanthosis) is the most common change associated with leukoplakia [23] and defined by a thickened epithelium without significant cytologic atypia. It is usually a benign condition, and it is peculiar but telling that

hyperplasia is listed as a cancer precursor lesion in oral cavity [502], whereas in most other squamous mucosae, this term is reserved for reactive alterations. Most leukoplakias are hypertrophic and therefore will have some degree of hyperplastic changes. By definition, the number of squamous epithelial layers either in basal/parabasal or superficial spinous layer is increased, and no cellular atypia is present [554] although the threshold for the latter determination is likely variable from one observer to other. Differential considerations include reactive changes (frictional keratosis) and mild dysplasia, the latter differential largely based on individual observer's preset threshold for defining cytologic atypia. Squamous hyperplasia without dysplasia has probably no or limited malignant potential, but follow-up of all leukoplakia lesions is mandatory.

Verrucous hyperplasia has, as previously described, a distinctly higher risk of transformation than regular acanthosis [293, 534, 563]. This lesion is not currently included in the latest WHO classification. Recently, an Asian consensus conference proposed criteria for oral verrucous papillary lesion as well as a new entity, exophytic oral verrucous hyperplasia (OVH), characterized by keratin plugging and epithelial dysplasia among other criteria [564]. This putative entity was diagnosed by one of the participants in an existing cohort. After re-review, a significant percentage of lesions had the original diagnoses changed: 18 VC (18/57; 32%)

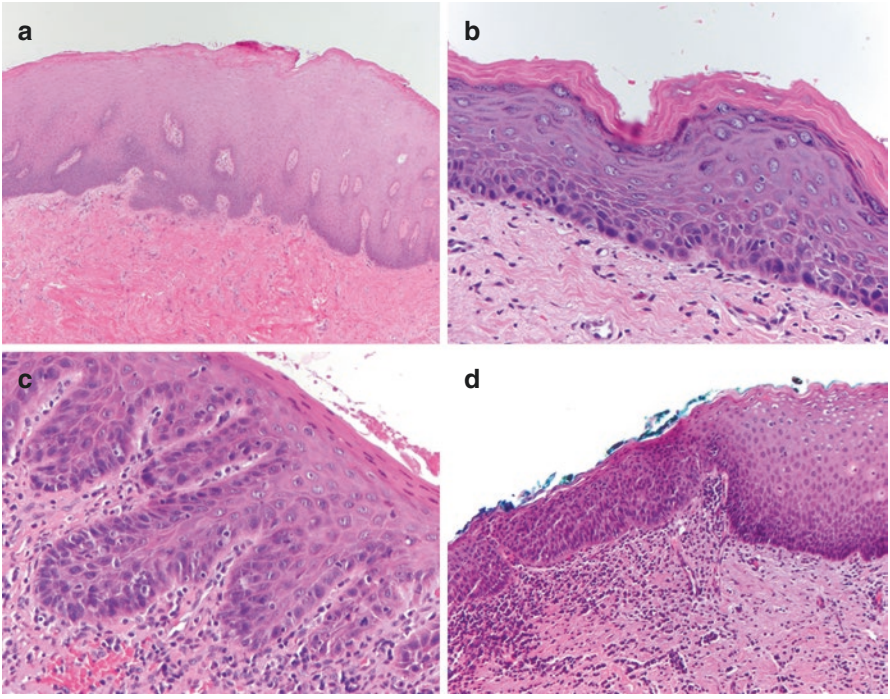

Fig. 5.16 (**a**) Dysplasia grading in potentially malignant disorders. (**a**) Hyperplasia without dysplasia. (**b**) Leukoplakia showing hyperkeratosis (*right* aspect) and mild dysplasia. (**c**) Moderate dysplasia with both architectural disorder and cytologic atypia. (**d**) Severe dysplasia/squamous cell carcinoma in situ adjacent to hyperplastic nondysplastic mucosa (*right*)

diagnoses were downgraded to OVH and 12 OVH diagnoses (12/38; 32%) were upgraded to VC. Interobserver variability and overlap with VC are clearly issues in these lesions. In addition, there are etiologic questions since more than half of these Indian patients had history of tobacco chewing. These authors proposed that OVH is a specific PMD in Asian (Taiwan, Malaysian, Indian) population, a precursor to VC and/or OSCC [565]. These lesions are indeed probably different from those described by Shear [534] since most patients have tobacco or areca nut chewing-related lesions and dysplasia [563, 565, 566]. Regardless of etiology, it is likely that VH and OVH (if truly different) are PMDs with higher transformation risk to either VC or SCC and their distinction from the former may be particularly difficult, likely due to a biologic continuum.

Mild dysplasia is defined by minimal architectural disorder, limited to the lower third of the mucosa, and associated with a degree of cytological atypia. In addition to reactive changes, distinction from moderate dysplasia can be difficult and is somewhat subjective. This diagnosis should probably not be made when numerous atypical mitotic figures or complex architecture with endophytic or angulated rete ridges are present (Figs. 5.16 and 5.17). Most mild dysplasias will likely regress or have no significant impact on survival. While no clear-cut guidelines exist, we do

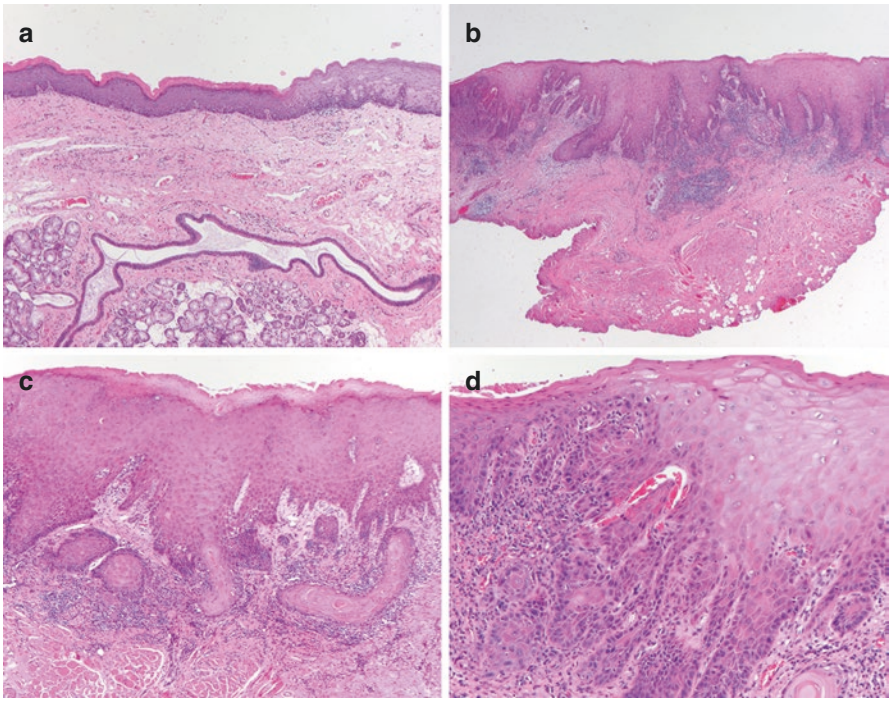

Fig. 5.17 Oral leukoplakia heterogeneity. Biopsies of this large, recurrent lateral oral tongue leukoplakia in a 48-year-old nonsmoker woman showed (**a**) areas of hyperplasia (*right*) and mild dysplasia with parakeratosis (*left*); (**b**) anteriorly, the same lesion showed multifocal microinvasive carcinoma (**b**) arising from mild (**c**) or moderate (**d**; *left upper*) dysplasia. There was no severe dysplasia or carcinoma in situ present

not report the presence of low-grade dysplasia at the margins either at the time of intraoperative consultation or in the final report in our practice.

Moderate dysplasia is defined by architectural disarray extending into the middle third of the mucosa with moderate cytologic atypia. An upgrade to severe dysplasia in cases with *marked* cytologic atypia even when limited to lower third is allowed in current WHO classification, lending additional subjectivity to the grading process [502]. This category is particularly clinically problematic since historically it has been grouped both under low-risk [23] and more recently high-risk lesions [554]. In research histopathology, cases of moderate dysplasia not uncommonly are divided between high and low risk almost equally and therefore may have a significant impact if a binary system is used for clinical management decision. In our laboratory and clinical practice, we include moderate dysplasia in the high-risk group [567] and report these changes both in frozen sections and in the excision specimen when present at the margins, strategy recently included in the College of American Pathology guidelines [11].

Severe dysplasia is defined by pronounced architectural disorder involving more than two-thirds of the mucosa with significant cytologic atypia. Unlike the changes seen in CIN3, there is often extensive maturation and keratosis toward the surface (Fig. 5.16). For this and other authors, oral squamous severe dysplasia is indistinguishable from carcinoma in situ in the vast majority of cases, and many pathologists consider (and report) them together in the malignant transformation risk spectrum [109, 308, 557, 560].

Carcinoma in situ is defined as "full or almost full thickness architectural abnormalities" with severe cytologic atypia and superficial mitoses. As previously mentioned, the "almost full" is the norm not the exception in oral dysplasia since most lesions exhibit at least a degree of superficial keratinization (i.e., maturation). Again, many head and neck pathologists find severe keratinizing dysplasia to be virtually indistinguishable from carcinoma in situ [308], and it is important for the treating clinician to understand that distinction is extremely subjective and the biologic risk of the former is probably not significantly lower than the latter, if different at all. In addition, CIS is seen in less than 20 % of mucosa adjacent to oral cancer [559] including very small tumors [25], and conversely, invasive tumors often arise in mucosa with low-grade dysplasia in a significant number of cases.

Unsurprisingly, currently there is no consensus on managing clinical dysplasia [568], since the level of evidence is low given the absence of randomized trials. Therefore, it is important for the treating clinician to understand the multifactorial origin of the current uncertainty surrounding the concept in order to integrate the oral epithelial dysplasia diagnosis in their clinical decision-making. Future practice changes which may improve the predictive value of dysplasia grade are going to be discussed. Several questions are currently unresolved which can be classified under three broad categories discussed below: (i) conceptual (is the wrong set of criteria used for oral dysplasia?); (ii) biological (molecular changes associated with dysplasia and carcinoma); and (iii) practical (interobserver variability, sampling of heterogeneous lesions).

5.3.5.1 Conceptual Problems

Conceptual problems in dysplasia grading are multifaceted and start with its definition: dysplasia is an artificial concept when the observer is asked to separate a disease continuum into discrete categories [569] and therefore by necessity a subjective exercise [570]. Moreover, the individual morphologic criteria proposed by WHO (Table 5.3) are probably accepted by most pathologists [554], but they may be expanded with additional architectural changes and their relative weight better defined. The emphasis on certain criteria with more predictive value should be translated into a scoring diagnostic schema. While WHO system is the most widely used, the precise combination and weight of each architectural and cytologic criteria for each grade is ill-defined and highly subjective [561, 571]. Recently, careful review of each of the WHO architectural and cytologic alterations [572] as well as additional ones [561] provided useful information on each criterion reproducibility and predictive value, but larger studies are needed for validation. For example, the presence and localization of superficial mitoses are critical in assigning cervical dysplasia grade, whereas we found them only in 27 % of the mucosae adjacent to and mitotic figures were entirely lacking entirely lacking in 35 % of a small oral cancer cohort [25]. The upgrade of mild or moderate dysplasia based on *cytologic* features is certainly justified but somewhat restrictive and eminently subjective. Of the seven architectural criteria listed by WHO (Table 5.3), only one addressed the downward growth (drop-shaped rete ridges). Additional useful architectural criteria including angulation, paradoxical maturation, horizontal growth, and complex, acute angle fusing are probably under-recognized in pathologic practice and recently tested by another group which found the WHO "thirds affected" or layer-based system unsatisfactory [592].

5.3.5.2 Molecular Aspects of Oral Dysplasia

There is an abundant body of literature on many molecular alterations in oral dysplasia that precede, harbinger transformation to, or are shared with, oral carcinoma. However, many of these genetic and epigenetic alterations were also reported in mucosa with low-grade or no dysplasia [573]. These genetic alterations have been recently reviewed and most were deemed to require additional validation, ideally in a prospective, multicenter study before clinical usage [568, 574, 575]. Several biomarkers are shared by dysplasia and carcinoma: loss of heterozygosity (most commonly in 3p and 9p loci), p53 overexpression, high proliferation index (ki-67, PCNA), retinoblastoma pathway/p16, tyrosine kinase pathways (EGFR, PI3K/AKT, ERK/MAPK), and cyclin D1, VEGF, and DNA content/aneuploidy, being the better studied [568, 574, 575]. Some of these markers have strong correlation with dysplasia grade (aneuploidy, p53, proliferation index), but evidence is currently insufficient for most markers' predictive value. Only a few were deemed ready for prospective validation before clinical use (3p and 9p, ERK/MAPK pathway alterations) when critically reviewed [575]. Epithelial-mesenchymal markers implicated in loss of cohesion and epithelial-mesenchymal transition, such as e-cadherin [577, 578, 582] and HMGA, are expressed earlier

than previously thought [579, 580] and may also potentially play a role in transformation although this requires confirmation in larger studies. Wide variation in the patient selection, sample size, type of biopsy and tissue available, pathology review, methodology employed, overall study design, follow-up, and treatment strategy hampers a meaningful comparison among different studies, which often have conflicting results and thus preclude definitive conclusions or general recommendation for clinical practice [568, 575]. Most importantly, the lack of standardization in clinical potential malignant disorder classification by the clinicians and histopathologic grading of dysplasia by the pathologists are major issues in validating these markers and identifying the potential malignant disorders with high risk for progression. Therefore, none of these markers have been validated or uniformly accepted for clinical practice to date.

The role of HPV in oral carcinogenesis is minimal as recently demonstrated by transcriptionally active virus (E6/E7 mRNA) detection in 0–5 % of OSCC [421, 425]; somewhat higher numbers were reported for PMD [552]. Earlier literature reports of higher prevalence of HPV in OED had discrepant results likely due to nonspecific methodologies [581, 582]. However, rare HPV-associated oral high-grade dysplasia does exist [583, 584], and a specific phenotype with increased apoptosis was recently described [583]. p16 expression was reported to be helpful in differentiating dysplastic from nondysplastic PMD [585], but when using stricter criteria for positive reaction similar to those currently accepted for oropharyngeal carcinoma, only 7 % of all oral dysplastic lesions were p16-positive and no predictive value for progression to carcinoma was detected in one study [552]. In addition, it is generally accepted that p16 reaction has low positive predictive value for HPV status in oral cancer [421, 425]. Since HPV positivity at oral sites has unclear biologic and clinical significance, routine testing is not recommended at this time for oral cavity squamous carcinoma or preneoplastic lesions [426].

5.3.5.3 Practical Considerations

Interobserver variability in assessing oral dysplasia grade may have been a surprise three decades ago when, after evaluating the same lesion, pathologists' diagnoses ranged from the benign to the malignant categories of the neoplastic spectrum in a landmark study from 1985 [570], but has been since well documented in multiple studies [571, 572, 586–591] and this remains a recurrent theme today in referral or consultation practice [453]. Its roots are complex, likely not restricted only to experience, subspecialty, imperfect criteria, and individual diagnostic threshold variance but also to the specific training of each observer [554]. Many studies showed poor or fair agreement between pathologists in grading oral epithelial dysplasia particularly at the low-grade part of the spectrum [571, 572, 586–590]. Some systems have better reproducibility than others [590], but intra-observer reproducibility issues are also common [587, 590].

One important cause for this lack of agreement may be related to the observer's training. Oral biopsies of potentially malignant disorders are reviewed and their dysplasia graded by oral pathologists, general surgical pathologists, and head and neck pathologists. While currently no data for each group preponderance in various regions can be found, and wide variability should be expected based on

different countries' health-care systems or even on local practice difference, it is likely that the surgical pathologists are going to be most likely involved in the diagnoses worldwide in general practice and, through dental practices, the oral pathologists will be receiving these samples quite commonly as well. Least frequent will be the large hospitals or tertiary care centers with a dedicated head and neck pathologist on staff, usually the case only in referral centers. Oral pathologists are by far the most common observers in most published research to date. Several studies found that oral and/or head and neck pathologists have higher interobserver agreement and lower threshold in diagnosing and grading oral dysplasia than general pathologists [571, 592, 593]. Differences in training of the oral and general/head and neck pathologists may play an important role in the discrepant rates: while the oral pathologists train, validate and calibrate their diagnostic criteria using mostly oral samples, the general pathologists train using the cervical squamous dysplasia paradigm, the most common squamous preneoplastic lesion available both in incisional biopsies and conization specimens in the general pathology services. Presumably the same paradigm is applied to the oral lesions by the general pathologist. The reasons that the cervical paradigm is not appropriate for oral preneoplastic lesions are well recognized for a long time [560]. Oral cavity dysplasia is different from cervical dysplasia in several etiologic, histopathologic, and biologic aspects. Firstly, oral dysplasia arises in *native* squamous epithelium and has a complex etiology, predominantly related to smoking and alcohol consumption; in contrast, the cervical intraepithelial neoplasia, which is almost exclusively human papillomavirus related, arises in the transition zone through a *metaplastic* process. Secondly, the progressive lack of maturation defines high-grade cervical dysplasia, whereas keratinizing dysplasia involving oral cavity resembles morphologically differentiated vulvar intraepithelial neoplasia much more than cervical intraepithelial neoplasia [559, 590]. Thirdly, localization of mitotic figures in the epithelial layers is a cornerstone of cervical intraepithelial neoplasia grading. In contrast, we have found mitotic figures in mid and superficial layers only in 20% of dysplastic mucosa adjacent to small oral cancers [25], and others have shown that presence of superficial mitoses and atypia has only fair to moderate interobserver variability [561, 572]. Finally, cervical carcinoma is rarely (if ever) associated with low-grade dysplasia only, whereas in oral cavity low-grade keratinizing dysplasia is commonly present adjacent to small cancer [25, 559, 560, 594]. In other words, cervical intraepithelial neoplasia progression to invasive carcinoma is predictable and reliably seen only from the highest end of the dysplastic spectrum (severe dysplasia/carcinoma in situ) in contrast with oral carcinoma which not uncommonly arises from low-grade dysplasia (Fig. 5.17).

This underlines the necessity of developing uniform standardized criteria and a dedicated grading system since many oral dysplasia samples may not be evaluated by a subspecialist. However, even when very experienced oral pathologists review the same cases, poor to fair interobserver agreement in assessing dysplasia grade was common [586, 589, 591], and the same samples can still be interpreted both as benign and malignant by two experienced observers occasionally [591] just like in Pindborg's landmark study in 1985 [570].

In an attempt to improve this issue, *a binary system* was recently proposed as an alternative that was shown to predict malignant transformation with 82 % accuracy [571] and has gained some acceptance, albeit not universal [554]. We have used such a system in our department both in clinical practice and for protocol purposes for almost a decade [567]. This practical system has been shown to decrease intra- and interobserver variability [571, 592, 593], better predict transformation than WHO system [550, 571, 592], and significantly help clinical stratification of these lesions: low-risk lesions (no/questionable/mild dysplasia) are clinically followed up and high risk (moderate/severe dysplasia/carcinoma in situ) are addressed clinically either surgically or with one of other several nonsurgical treatment modalities available. In this system, lesions with four or more architectural and five or more cytologic alterations were classified at high risk for malignant transformation [571] and subsequently better interobserver agreement confirmed by a different group [592]. Interestingly, in this latter study, one-third of moderate dysplasias progressed to carcinoma and the difference between the lesions classified as low risk from this subgroup was not significantly different from the lesions classified as high risk in the binary system in the same subgroup (moderate dysplasia). Unweighted addition of various parameters is problematic in this system, but that is probably true for any system trying to quantify a number of subjective assessments [561]. Nevertheless, even if moderate dysplasia continues to present challenges in this classification, being about equally split between low- and high-grade categories [572, 592, 593], the binary schema may be more practical for clinical management and provide a uniform schema for clinical trials.

Currently consensus recommendations for dysplasia management do not exist, precluded by lack of data [568]. The binary system has been recently gaining more acceptance in consensus papers and various clinical guidelines [11, 554], and some variation of it has been already employed in research studies by several groups [500, 550, 567, 578]. A multicenter validation of this system is desirable before clinical implementation.

Sampling Differences PMDs are renowned for their heterogeneity as illustrated in Fig. 5.17, and suboptimal sampling may provide the explanation for lower predictive value of oral dysplasia compared to other mucosal surfaces. Sampling of large lesions is inherently biased and will affect grading predictive value (Fig. 5.17) [36].

As discussed, this includes clinical sampling of the lesion or lesions, technique used, number and size of biopsies, and quantity of mucosa represented. In speckled leukoplakia, the biopsy should be targeted at the red areas. Correct processing and embedding of oral biopsies in the pathology department and obtaining step sections on each block will increase the chance that the pathologist reviews under the microscope the worse lesion or area and is able to accurately score the highest grade dysplasia.

5.3.5.4 Early carcinoma

Early carcinoma includes microinvasive and superficially infiltrating carcinoma, terms often used interchangeably in the literature. A distinction should be made since the former carries no or minimal risk for metastatic spread, whereas the latter has slightly increased risk. Early OSCC presents clinically as a PMD, most likely nonhomogeneous leukoplakia [24, 25], but even in homogenous leukoplakia, subclinical cancer was found in 7 % of cases in one large series [36].

Preneoplastic lesions are defined by preservation of an intact basement membrane, whereas cancer overcomes this barrier involving the lamina propria and submucosa. This structure is not always well defined [560], and the transition from the noninvasive to the invasive stage of the neoplastic process is fraught with interobserver variability and is probably one of the most common disagreements in the field based on my personal experience (Figs. 5.1 and 5.12). The discrepancy will increase when erosion or ulceration exists, a dense lymphoid infiltrate is present at the interface, or when dysplasia extends into salivary ducts.

When does the earliest stromal penetration occur can be unclear. Often times, continuity of dysplastic epithelium to the surface mucosa precludes the pathologist to establish the presence of invasion, a traditional and conservative point of view [560]. We do not subscribe to that definition and do not require that the tumor cells "are surrounded by stroma" in order to establish invasion (Fig. 5.18). Depth of involvement, architectural complexity, with angulated branching and presence of epithelial tongues into deep submucosa may suffice. In the end, all early invasive

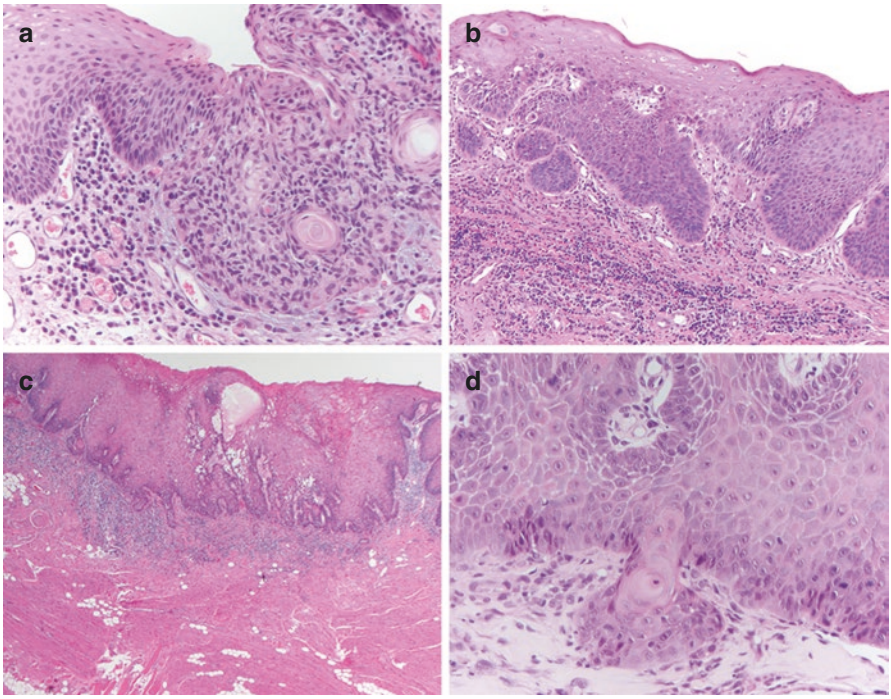

Fig. 5.18 Early squamous cancer is often associated with low-grade dysplasia and is contiguous to the surface. (**a**) This microinvasive carcinoma of oral tongue (*center low*) with minimally dysplastic adjacent mucosa elicits desmoplastic response. (**b**) Despite surface maturation, the drop-off dysplastic epithelial projections often harbinger early stromal invasion. (**c**) Early invasion arising in low-grade dysplasia is seen in a broad oral tongue leukoplakia and associated with a band-like lymphoid response. (**d**) Paradoxical maturation below basement membrane and abrupt lamina propria vertical penetration are the earliest signs of infiltration in this hard palate erythroplakia with extensive keratinizing dysplasia

carcinomas have contact with the surface mucosa from which they arise if a three-dimensional image would be obtained.

Microinvasive carcinoma is currently lacking not only a qualitative definition but also a uniform quantitative minimal threshold for those observers and authors who would prefer a measurement objectivity. Measurement cutoffs from 0.5 cm to 2 mm for depth of invasion [1, 308, 560] and 4 mm for tumor thickness [24] were proposed in the literature to define microinvasive carcinoma, while others use only the histologic landmarks to characterize early carcinoma, further divided into microinvasive and superficially invasive based on the depth of involvement relative to rete ridges, but confined to mucosa without extending into submucosa [63]. It is not surprising that controversy exists when a carcinoma is *micro*invasive and *superficially* invasive or has frank invasion [24, 595]. By definition, tumors with lymphatic or muscle invasion should not be considered microinvasive regardless of depth [308].

This is obviously not an academic distinction since establishing the presence and significance of incipient stromal involvement in PMD would provide a theoretical framework for both treating local disease and managing the neck. There is tremendous variation in cutoff measurements, but 1.5–2 mm depth and 4–5 mm thickness are most commonly used [24, 560] as discussed earlier – please refer to tumor depth and thickness section. Subsite differences are likely to exist. Uniform definition and consensus regarding reference points for TT and DOI measurement are currently lacking making thus a literature comparison virtually impossible [167].

Unfortunately, the final depth cannot be ascertained in the presurgical sample unless it is a large, excisional biopsy and it is not uncommon in large PMD with extensive high-risk dysplasia to observe several microinvasive foci separated by dysplastic or even normal-appearing mucosa (Fig. 5.17). We prefer the designation "at least microinvasive carcinoma" for biopsy specimens, which cannot reliably predict depth of invasion or severity of the lesion [560, 562].

In the absence of strict criteria, the diagnosis of microinvasive carcinoma remains a judgment call in most cases [596], and clear consensus on the terminology used (microcarcinoma, early invasion, superficial invasion) will be essential for clinical management in the future. A recent study of early vulvar carcinoma, tumor with many histologic and clinical similarities with oral cavity neoplasms, reported a worrisome interobserver variability among experienced specialty pathologists in both identifying the invasion and measuring its depth [597]. We have recently noted similar variance even when all measurements were performed using the same instrument from the same deep point [169]. As discussed earlier, clear definition, separation, and uniform reporting of tumor thickness and depth of invasion are essential to quantify the risk for nodal involvement. Microinvasive carcinoma has an excellent chance for cure by excision which usually suffices, and it should be separated from superficially infiltrating cancer.

Since detection and treatment of oral cancer at its earliest stage is key for curative management, prospective, multicenter studies are needed to elucidate where does the clinically relevant stromal involvement occurs and define the optimal cutoff thickness. Until then, careful perpendicular sectioning, clearly reporting whether depth or thickness is measured, and local cooperative effort by surgeons and pathologists are essential for uniform clinical management.

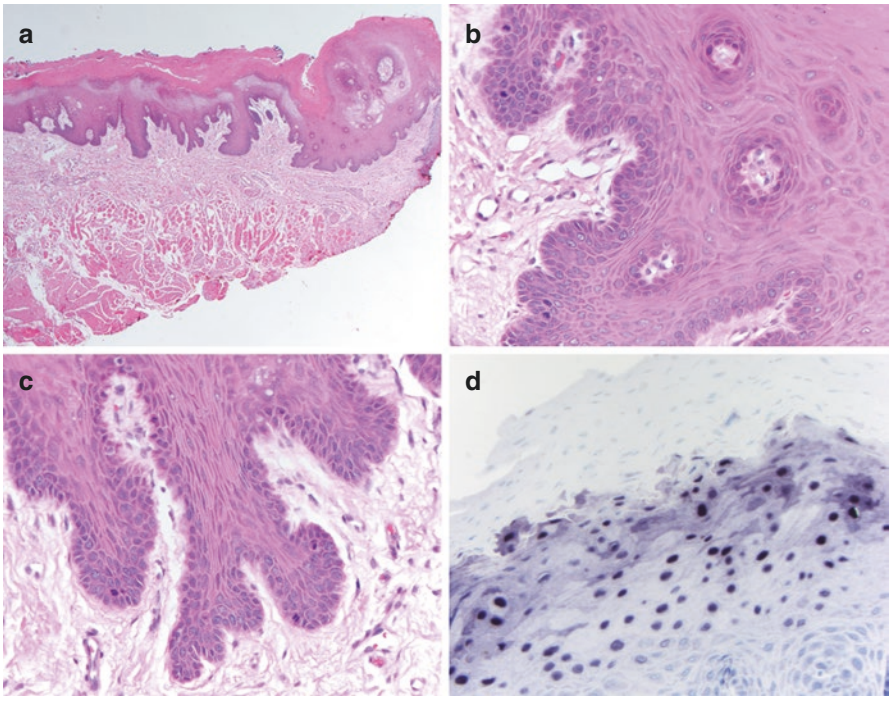

Fig. 5.19 Hairy cell leukoplakia. This 65-year-old man presented with lateral tongue nodular leukoplakia, growing fast for several months. (**a**) This proliferative keratotic process showed spinous layer clearing ("balloon changes") and focal complex architecture and basal atypia with increased mitoses (**b**, **c**). Dysplasia was initially considered but Epstein-Barr virus testing (EBER, **d**) confirmed the diagnosis of hairy cell leukoplakia, a lesion without malignant potential but mandating immune status evaluation

In summary, oral cancer can arise from minimally dysplastic or even normal mucosa. Grading dysplasia remains the most powerful predictor of malignant transformation, but PMD size, appearance, and location are important. Standardization of terminology for both clinical and histopathologic evaluation and classification of PMD is essential for prospective, multicenter collaborations to identify and validate novel markers.

5.3.6 Oral Epithelial Dysplasia and Early Oral Cancer Diagnostic Pitfalls

Several infectious diseases oral manifestations including, but not limited to, Epstein Barr virus-related hairy cell leukoplakia (Fig. 5.19) and various *Candida* spp.-associated hyperplastic (Fig. 5.20) or erosive lesions can mimic PMD both clinically and histologically. In oral candidiasis, lesions may appear grossly white or red (erosive), and microscopically the epithelial atypia can appear dysplastic. Chronic

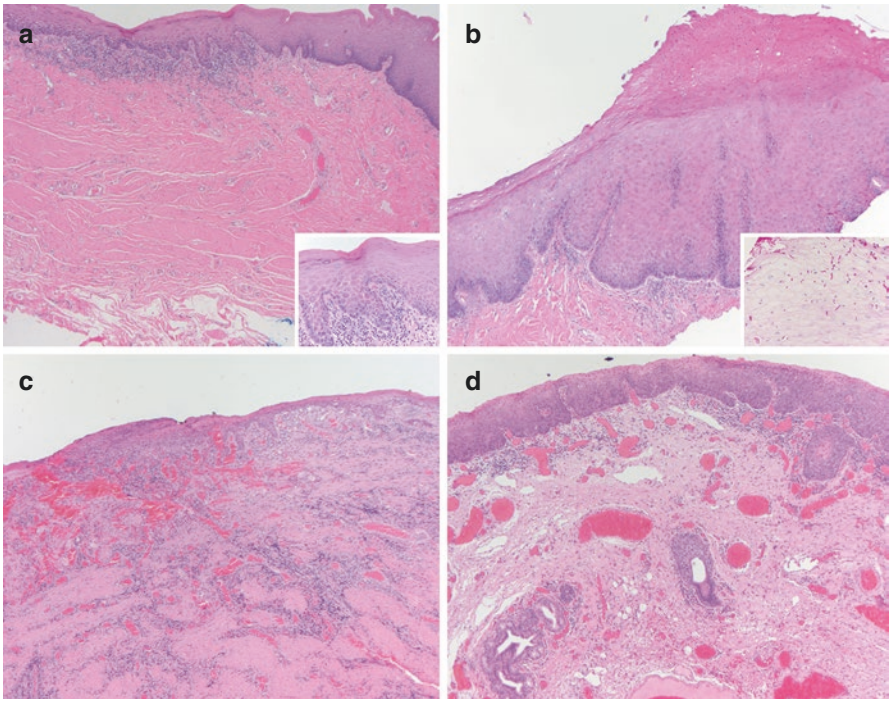

Fig. 5.20 (**a**) 'Lichenoid' mild dysplasia (*left side*; higher magnification *inset*) adjacent to normal mucosa (*right*). (**b**) This tongue leukoplakia with mild dysplasia showed multiple *Candida* organisms in the keratotic layer (*right upper*), best seen with periodic acid Schiff (*inset*). Rebiopsy after treatment may be recommended in such cases. (**c**) Erosive lupus may clinically mimic erythroplakia and associated cytologic atypia misinterpreted as dysplasia. (**d**) Severe dysplasia tracking along the minor salivary duct should not be interpreted as invasive carcinoma but may be the root of local recurrence

hyperplastic candidiasis is considered by some authors preneoplastic, but to this observer, it is unclear whether dysplasia seen in many such cases is the cause or the consequence of the infection (Fig. 5.20). Reevaluation after treatment is advised.

Oral lichenoid lesions can mimic dysplastic lesions and encompass a broad spectrum of conditions, but given their proven higher risk of transformation than oral lichen planus, a subset probably represent true epithelial dysplasia. Conversely, a lymphoid infiltrate restricted to areas with high-grade dysplasia or microinvasive carcinoma but absent from the areas with lower-grade dysplasia was noticed by this observer in a subset of OLLs and may be a harbinger for impending stromal invasion. Step sections may be helpful in identifying the invasive focus (Figs. 5.1, 5.2, 5.14, 5.17 and 5.18). Other erosive inflammatory conditions such as lupus mucositis may be associated with significant atypia and be misinterpreted as dysplasia (Fig. 5.20).

Hyperplastic lesions with no or low-grade dysplasia tangential (horizontal) embedding can mimic a higher-grade dysplasia or even superficial invasion. Extensive dysplasia in a single broad preneoplastic lesion can encompass variable

degrees of dysplasia, from mild to severe, or even to carcinoma in situ and microinvasive cancer as illustrated in Fig. 5.17.

Microinvasive or early invasive carcinoma may be underrecognized, particularly when arises in the background of low-grade dysplasia and when the "invasive island" separation criterion is strictly enforced. When ductal extension of high-grade dysplasia/carcinoma in situ is present [63, 598, 599], stromal involvement at distance from mucosal surface could lead to local recurrences, as it is well described in cervical intraepithelial neoplasia. Misinterpretation of intraductal extension of squamous dysplasia should be avoided, since it may lead to unnecessary local or nodal treatment (Fig. 5.20).

Other potential mimickers of squamous dysplasia include pseudoepitheliomatous hyperplasia, mucosa adjacent to osteonecrosis, or other ulcerative lesions in oral cavity, previously discussed under squamous cell carcinoma section.

5.3.7 Potentially Malignant Disorders: Summary

Potentially malignant disorders are a heterogeneous group of oral lesions and conditions with variable risk for malignant transformation. A few longitudinal and no randomized studies exist to date, and therefore no consensus guidelines are available for clinical practice.

Large, heterogeneous lesions, female gender, and presence of dysplasia were found to harbinger the highest risk. The rate of transformation increases with dysplasia grade. A uniform clinical system for classification would be desirable for research protocols and clinical purposes, and a binary dysplasia grade system may increase interobserver agreement and predicting value.

An ideal scoring algorithm of PMD would include all variables reported to be of prognostic importance including size, site, clinical appearance (using a uniform designation), histologic grade, focality, and other host risk factors. Comparing these lesions in a prospective, multicenter study would be needed to quantify the risk of various clinical and histologic parameters and allow novel marker development. Creating a modular prognostic nomogram for PMD should be the goal for uniform assessment and risk stratification.

Separating the high-risk from the low-risk lesions provides a practical clinical framework to identify the patients at high risk for transformation, allowing detection and management of early carcinoma and improving thus patients' outcome.

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Emerging Cancer Biomarkers for HNSCC Detection and Therapeutic Intervention

6

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6.1 Introduction

Globally, cancer remains a major public health problem. In spite of the many advances in our understanding of cancer as a disease, and in prevention and treatment strategies, it is estimated that there will be ~12 million newly diagnosed cases, and more than 7 million are expected to die (about 13 % of all deaths), making cancer a leading cause of death worldwide [52]. These numbers are projected to reach approximately 26 million new cases and 17 million deaths by 2030 [134]. For United States alone, one in four deaths is attributed to cancer, and with 1,638,910

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new cases and 577,190 deaths expected for 2012 [119, 120], it will surpass heart disease as the number 1 cause of death, reflecting the indiscriminate nature of cancer irrespective of country borders or socioeconomic status [122]. Among the 10 most common human malignancies, an estimated 263,900 new cases and 128,000 deaths involve oral cavity cancers [52]. Approximately ~90 % of these lesions are of squamous cell origin and often referred to as head and neck cancer (HNSCC), which account for more than 500,000 new cases and 250,000 deaths every year when squamous carcinomas of the pharynx are considered together with those arising from the oral cavity. In some Southeast Asian countries, its high occurrence makes HNSCC the top cancer in men [52]. Currently the worldwide rising incidence of human papillomavirus (HPV)-related oropharyngeal cancer is alarming and a cause for concern [20]. In general, HNSCC is associated with poor prognosis due in part to the lack of suitable markers for early detection, and hence its late diagnosis when either the lesions are well advanced or have already metastasized to regional lymph nodes, thus compromising patient care and likely therapeutic outcome [65, 69]. Therefore, accurate approaches for the timely diagnosis of HNSCC both at early and advanced stages are critical to better assess treatment options and improve patient outcome [1, 100, 124]. In this endeavor, identifying relevant biomarkers for use in (1) developing diagnostic and prognostic tools, (2) early-stage disease prediction, (3) patient stratification for more effective and targeted treatments, and (4) identification of those patients most likely to relapse or succumb to treatment failure becomes an attractive proposition that will surely help to reduce cancer burden and mortality [135]. In this chapter, we will describe several initiatives that are likely to advance our understanding of HNSCC as a disease and in the process help to increase our discovery pipeline of cancer biomarkers as well as describe some notable examples of those that are already making an impact in the clinic.

6.2 Cancer Biomarkers

The National Institutes of Health (NIH) defines biomarkers as molecules that can be reliably and accurately measured and are indicators of normal or disease biological processes and responses to therapeutic interventions [10, 77]. These biomarkers can include physical symptoms, secreted proteins, mutated DNAs, altered mRNA levels, or concentrations of small molecules in serum and saliva, to name a few. In cancer, the single most important goal of a biomarker is the reliable detection of the presence of the smallest number of tumor cells before further growth, when clinical outcome and prognosis are still favorable for the patient [74]. Levels of biomarkers are generally low in the body, fluctuating marginally between a narrow range during the onset of cancer and while the presence of a marker may not be causal to the underlying disease; nevertheless, understanding the mechanism responsible for the appearance of the marker can help in determining its specificity for cancer detection [50]. A challenge, though, is how to reliably identify and measure suitable biomarkers, ideally using noninvasive approaches that can further increase sensitivity

(defined as the ability of an assay to identify a condition when it is present) and specificity (rule out a condition when it is absent) and with high positive and negative predictive values. Collectively, both should reach >90 %, to avoid false positives and false negatives, which is crucial for avoiding misdiagnosis and improving decision-making abilities [49, 63].

Ideally, cancer biomarkers should reflect specific stages of the disease, so they may be developed for use in diagnosis and prognosis, predicting cancer therapy and treatment efficacy [51, 118]. However, single biomarkers alone are often unlikely to provide the necessary predictive values needed for reliable cancer detection and monitoring. For example, prostate-specific antigen (PSA), an FDA-approved and first clinically used single protein biomarker for prostate cancer, has a positive predictive value of ~70 %, and while this assay can broadly detect the cancer, the potential for misdiagnosis still remains, resulting in unnecessary medical treatment and stress [77, 127]. It follows that a collection or panel of biomarkers can provide a superior predictive power, and measurement with equivalent accuracy of elevated levels of 4–10 distinct molecular markers for a given cancer should provide a much better statistical basis for successful prediction [50, 58]. Therefore, a panel of cancer-specific biomarkers ideally would be required to accurately and reliably detect the cancer, for point-of-care diagnosis and better understanding of the disease [112, 113].

The road to biomarker discovery, validation, and US Food and Drug Administration (FDA) approval, for inclusion into a panel of makers, is faced with several challenges, including long time taken, financial cost, and the attrition rate. Notwithstanding, advanced biomarker-based technologies, for example, by the use of high-density whole-genome microarrays and combined with several next-generation-based sequencing (NGS) platforms and data analysis, are well suited for comprehensive genome-wide profiling of tumor biopsies, with the goal of identifying more efficiently novel targets as viable biomarkers for clinical use [111]. From these approaches, targets can include those constituting aberrant signaling pathways that are important for cell proliferation and gene alterations (such as point mutations, deletions, amplifications, translocations), which are now known to directly contribute to the abnormal growth of the cancer cell. Furthermore, the presence or absence of mutations within these genes may also have predictive value for response to a specific targeted therapy, which could be developed into clinically viable biomarkers, with several high-profile examples of these categories of markers highlighted below [42, 83]. On the other hand, gene signatures of tumor biopsies have excellent value as classifiers. Earlier work has empowered the identification of prognostic subclasses of cancers previously undetected by conventional histological analysis, and highlighting the existence of a greater diversity in malignant diseases than previously anticipated [44, 140]. Some notable examples include diffuse large B-cell lymphomas (DLBCL), as data from earlier expression analysis was able to stratify two forms of this histological similar cancer, and identified a subtype in patients that conferred an overall superior survival. This seminal study highlighted the value of identifying a small number of marker genes that can be readily translated for clinical use to discriminate between histologically similar Burkitt's

lymphoma and DLBCL, to improve the therapy and patient stratification for clinical trials [2, 73]. Furthermore, gene signature panels are now being used clinically for the molecular classification of breast cancer for their prognostic prediction value in patients with early-stage breast cancer at risk for relapse, consequently influencing therapy [108]. To this end, the FDA now has a set of guidelines to help accelerate the approval of anticancer drugs that are based on markers that might predict clinical benefit, and consequently there is now a strong emphasis on identifying and validating cancer biomarkers for guiding such therapies [82].

There are now several noteworthy examples of biomarkers in clinical use that are revolutionizing the way cancer patients are diagnosed and treated. Many of these therapeutic targets with their intended therapies are illustrated in Fig. 6.1. These include overexpression of estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2) in breast cancer, EGF receptor (EGFR) overexpression, the presence of a fusion between the echinoderm microtubule-associated protein-like 4 (*EML4*) gene and the anaplastic lymphoma kinase (*ALK*) gene in lung cancer, and the presence of activating mutations in *BRAF* (melanoma) and *RAS* (colorectal cancer) genes. Identification of these aforementioned genetic alterations has now led to the early accelerated FDA approval for highly targeted cancer drugs. For example,

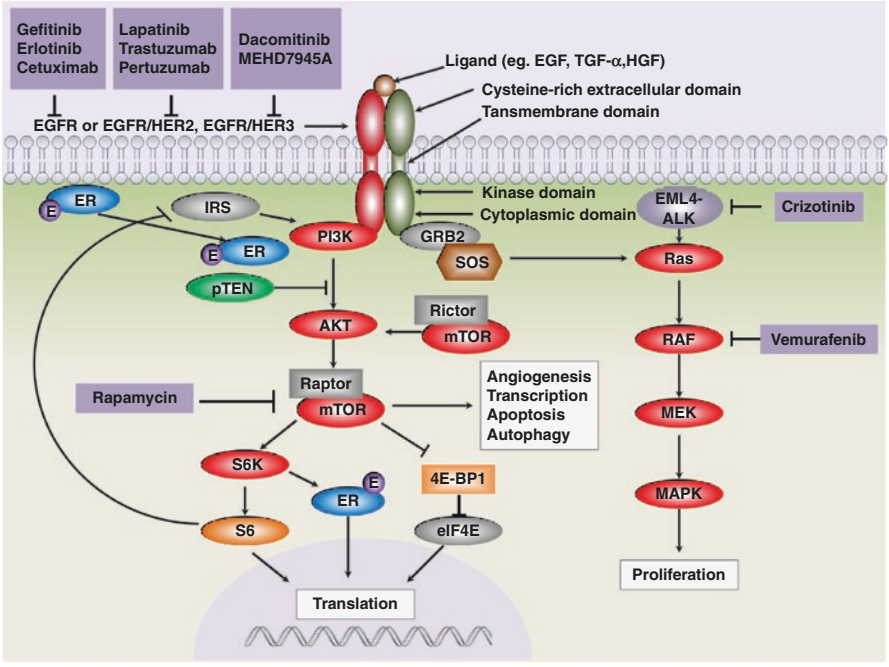

Fig. 6.1 Viable molecular targets for cancer therapies. Recently identified alterations in key molecules serving proliferative and survival signals in several different cancer types (breast, colorectal, HNSCC, lung, melanoma) have led the way for their development as predictive biomarkers for guiding targeted cancer therapies

overexpression of the HER2 protein in breast cancer is now used to predict which patients are most likely to benefit from receiving humanized monoclonal antibody therapy targeting HER2, such as trastuzumab that binds selectively to the HER2 protein and pertuzumab that inhibits the dimerization of HER2 with other HER receptors. This approach has now been shown to offer significant improvements in progression-free survival [25]. More recently, an antibody-drug conjugate consisting of trastuzumab and DM1 (trastuzumab emtansine, T-DM1), a potent antimicrotubule agent, tested in phase 2 trial gave favorable survival in patients diagnosed with metastatic and HER2-positive breast cancer, remarkably with minimal adverse toxicity largely due to internalization of the conjugate. DM1 targets tubulin and inhibits cell division of HER2 overexpressing cells only, without effecting normal cells [14]. These encouraging observations led to a large multicenter phase 3 trial (EMILIA Study), whereby 991 previously treated patients with advanced HER2-positive breast cancer were assessed for response to T-DM1, and at conclusion, the study found that this therapy offered an overall improved survival which was attributed as mentioned above to the intracellular delivery of the drug specifically to the tumor cells [130]. Furthermore, a two-pronged (2-in-1) attack on advanced ER+ breast cancer is now advocated, largely due to the upregulation of EGFR and HER2 proteins and resistance to tamoxifen. Lapatinib, a small molecule inhibitor targeting both receptors, has been shown to restore sensitivity to antihormonal therapy and essentially marks a new era of multi-targeted single agents [22, 45]. In a similar way, MEHD7945A is currently under evaluation as a 2-in-1 antibody targeting both EGFR and HER3 for inactivation, and emerging data suggests that this type of therapy may help overcome resistance to monospecific antibodies that essentially target individual receptors such as EGFR [114]. Following in this line of multi-targeted monotherapies, dacomitinib, a second-generation small molecule pan-HER inhibitor developed to combat resistance inherent to monotherapies against single targets, has shown promise in HER2-amplified gastric cancers, suggesting a better therapy option rather than using combination of different inhibitors [91]. These studies highlight the urgency for developing suitable biomarkers predicting the appropriate therapeutic option based on the members of HER family expressed in each tumor type.

The identification of EGFR tyrosine kinase domain mutations (exons 18–21) in a subset of non-small cell lung cancers (NSCLC) overexpressing the receptor predicted primarily those patients whose tumors harboring such mutations would benefit the most from treatment with small molecule inhibitors to EGFR (gefitinib and erlotinib), replacing cytotoxic chemotherapy [144]. Sadly, most patients on this predictive regime relapse within 1 year. However, a recent study elegantly demonstrated that circulating tumor cells from blood of lung cancer patients on anti-EGFR therapy could be isolated on micro-posts decorated with EpCAM antibody within a microfluidic device, and the DNA from the isolated cells can then undergo *EGFR* mutational analysis. In addition to detecting the activating mutation, the study identified a T790M mutation that matched to drug resistance and in the process those patients most likely to relapse [75].

Needless to say, the companion diagnostic kits for biomarker detection to guide cancer treatment in a clinically relevant timeline are gaining favorable regulatory

approval and represent a move forward from the blanket approach of cytotoxic therapies [55]. For example, the concurrent approval of crizotinib, a small molecule inhibitor targeting the aberrant form of anaplastic lymphoma kinase (ALK), and a companion diagnostic test to detect *EML4-ALK* genes rearrangement (Vysis ALK Break Apart FISH Probe Kit: Abbott Molecular, Inc.), found in about 4–5 % of patients with non-small cell lung cancer, mark yet another paradigm shift in the care and management of lung cancer. @@@Aligned with this, several diagnostic kits offering personalized healthcare are now available, to provide information such as mutational status of *EGFR*, *KRAS*, *BRAF,* and *PI3K* genes [43], to help predict patients most likely to benefit from a particular type of treatment. For example, melanomas with *BRAF* V600E mutation are likely to respond favorably to its inhibitor vemurafenib [11]. Other highly prevalent cancer types, such as prostate cancer, still hold a certain dilemma for physicians, with a need for novel molecular markers differentiating aggressive from nonaggressive prostate cancer, and biomarkers identifying patients having fast-growing malignancies which should be treated aggressively versus those who can be directed to active surveillance [26]. Recently, a study using a FISH probe to detect rearrangements of *ERG* and *ETV1* genes, and to measure loss of the *PTEN* gene, found that men with prostate cancer that had the alteration but no *PTEN* loss fared better with prolonged survival than those with *PTEN* loss without rearrangement of *ERG* and *ETV1* genes. Consequently, these markers can be used for predicting a group most likely to benefit from aggressive therapy [107].

The realization of existing genomic information can also impact the biomarker pipeline, leading to the development of technologically and innovative approaches for potential clinical application. In this regard, the National Cancer Institute-sponsored Cancer Genome Atlas (TCGA) project, with the single goal of cataloging the genetic encryptions of approximately 500 cases of each of several different confirmed cancers, now provides freely this vast resource of information to the scientific community [17]. Perhaps crucial for validation purposes, the large number of cases per cancer provides adequate power to correctly detect driving mutations that are now known to occur at a frequency of 3–5 % and, by falling within the range of sensitivity of TCGA, ensures that the emerging genetic information is of the highest standard and quality [17]. While the TCGA information can undoubtedly identify genetic alterations with therapeutic potential, validation studies to ensure the molecule's specificity to the underlining cause of the cancer are needed, and the nature of these types of studies can lead to creative and novel methodologies for assessing the biological consequence of the altered molecules [18]. In this regard, the detailed analysis of the available data for ovarian cancer and the use of a genome-wide short hairpin RNA (shRNA) screen identified that the inhibitor of DNA binding 4 (ID4) is important for conferring proliferative and survival advantage [109]. Additional studies to confirm the specificity of this molecule resulted in the development of a tumor-penetrating nanocomplex consisting of ID4-specific small interfering RNAs (siRNA) coupled with a tandem tumor-penetrating and membrane-translocating peptide (Ly-P-1) with a high affinity to an overexpressed mitochondrial protein. This essentially allowed for the targeted delivery of the siRNA to the tumor cells,

and when assessed in xenograft models of ovarian cancer, reduced tumor burden and increased survival were evident [109].

Noteworthy, in addition to the aforementioned, the emerging information from TCGA not only identified four primary subtypes (HER positive, luminal A, luminal B, basal-like) of breast cancer, each with its own distinct biological and clinical characteristics, but it was the genomic similarities between the basal-like subtype and serous ovarian cancer that unexpectedly raised a certain level of interest as both tumor types may be sensitive to the same treatment regime [16]. Critical information may also be gained from well-designed and executed experiments with extensive validation using standardized platform and protocols. Exemplifying this is a recent expression analysis performed on a large NSCLC clinically annotated sample cohort, which found a short list of viable biomarker candidates with potential prognostic value. Further evaluation by meta-analysis of relevant publicly available datasets essentially identified that high CADMI levels were found to be associated with improved survival and may represent a clinically relevant prognostic marker directly impacting survival and therapy of NSCLC patients [12]. Noteworthy, these valuable validation studies were performed using an antibody identified from the Human Protein Atlas, which is a publicly available database of high-resolution images of antibody directed expression patterns of protein of interest in several different normal and diseased tissues, thus allowing for a rapid assessment for the suitability of CADM1 as a suitable prognostic marker. Collectively, with these small incremental steps described above, huge advances have been made for the realization of predictive and precision cancer therapies [128].

6.3 HNSCC Biomarkers

Specific biomarkers for HNSCC do not exist yet for routine clinical use, but recent observations have provided several antibody-driven protein makers of interest that are currently under evaluation for their predictive potential. These biomarkers are being implemented into clinical practice, whereby newly diagnosed HNSCC cases that undergo routine H&E-stained histopathological analysis to provide information essential for TNM (tumor-node-metastasis criteria) classification could also be evaluated for some of these key predictive biomarkers within the same workflow, to aid, for instance, in treatment options [41, 70, 121]. The high-risk human papillomavirus 16 (HPV16) is now known to be highly associated with an increased incidence of oropharyngeal tumors in younger patients, and approximately 20 % of HNSCC are likely HPV+ [46, 62]. With the same standard of care, the response and survival of HPV+ patient group is overall superior to the HPV group, indicating that screening all newly diagnosed HNSCC for the presence of HPV16 may help in predicting the correct treatment. This may prevent the overtreatment of HPV+ HNSCC patients, where less aggressive and toxic targeted therapeutics could be considered as a primary option, rather than the more intense chemoradiotherapy-based approach, which is the current standard of care [4, 142]. In this regard, several studies including ours have shown that HNSCCs are largely associated with the

persistent activation of the AKT/mTOR pathway [3, 29, 99] and have extended this observation and demonstrated that HPV-associated HNSCC lesions were no exception and that rapalogs (e.g., rapamycin and RAD001) effectively decreased mTOR activity *in vivo*, resulting in a remarkable decrease in tumor burden. These studies provided a rationale for the clinical evaluation of mTOR inhibitors as a molecular-targeted approach for the treatment of HPV-associated HNSCC [87]. Activated AKT/mTOR pathway in HNSCC may be further exploited for identifying promising biomarkers. Indeed, recent studies have shown that histological overexpression of eukaryotic initiation factor 4E (eIF4E), which acts downstream of mTOR, is widespread in HNSCC and represents an independent predictor of recurrence in tumor-free surgical margins, identifying HNSCC patients who may benefit from additional therapy and notably allowing surgical guidance for tumor-free margins [92]. Aligned with this, aberrant accumulation of the phosphorylated active form of S6, p-S6, the most downstream target of the Akt-mTOR-p70-S6 kinase pathway, is a frequent event in clinical specimens from patients with HNSCC and in HNSCC xenograft models [3, 86]. Upon administration of clinically relevant doses of rapamycin (and rapalogs), p-S6 was dramatically reduced, providing the basis for screening newly diagnosed HNSCC patients for p-S6 status. This biomarker may predict which patient group will benefit from receiving mTOR-targeted therapy and, as a molecular endpoint, to predict treatment efficacy in patients undergoing treatment [93].

As mentioned earlier, the spread of primary HNSCC lesions to the locoregional lymph nodes has often already occurred at the time of diagnosis, and prognosis and long-term survival of HNSCC patients is compromised [65]. However, clinical assessment by physical examination and different imaging modalities, as well as by histological examination of routine lymph node H&E-stained cryosections, can miss micrometastases, while false positives may lead to unnecessary elective lymph node neck resections. Collectively, patients may miss the correct therapeutic window due to misdiagnosis [70]. We and others have shown that desmoglein 3 (DSG3), a transmembrane glycoprotein, is highly expressed exclusively in stratified epithelium, notably in normal oral squamous mucosa, and in all HNSCC lesions and their metastatic cervical lymph nodes at the mRNA [35–37] and protein [100] levels. As indicated (Fig. 6.2), DSG3 expression was seen only in invaded lymph nodes (N+), while N− was largely negative. Noteworthy, tumor cells in N+ were confirmed by H&E and cytokeratin staining. Therefore, the presence of DSG3 protein in cervical lymph nodes can serve as a sensitive marker of HNSCC progression for the detection of micrometastatic lesions and for the development of point-of-care clinical screening protocols to identify HNSCC patients with metastatic disease. To this end, we have exploited these observations, combined with the availability of highly specific monoclonal antibodies detecting different epitopes in DSG3, and our recently established biomarker detection platform using microfluidic immunoarray devices featuring nanostructured electrodes (covered in detail below), to develop an assay system enabling the rapid and ultrasensitive detection of DSG3 protein in complex tissue extracts, with minimal nonspecific binding. The method was found to be sensitive enough to detect isolated tumor cells, in a single cryosection of a

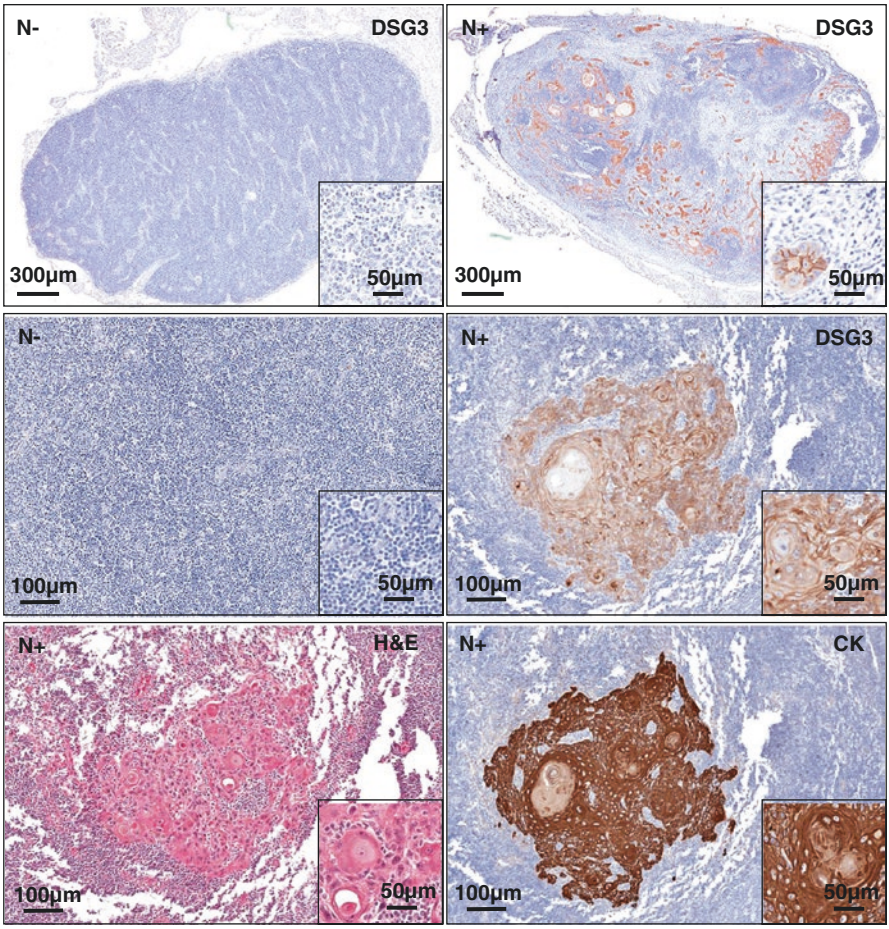

Fig. 6.2 DSG3 is expressed in invaded cervical lymph nodes. Archived tissue sections of non-metastatic (N–) and metastatic lymph nodes (N+) show DGS3 expression only in N+, with the staining localized to the malignant squamous cells (*upper panels*). At high magnification, small metastasis is clearly visualized by DSG3 staining in N+, while none were detected in the negative counterpart (*middle panels*), and the presence of squamous tumor cells was further confirmed by cytokeratin immunoreactivity (*bottom panels*) (Data are from Patel et al. [100])

positive lymph node, and help predict the diagnosis, guiding in the selection of appropriate therapy for HNSCC patients [100]. Other classes of protein biomarkers include cyclin D1, a molecule involved in cell proliferation, and overexpressed in many HNSCC, which is closely associated with cisplatin resistance and poor outcome [90]. A recent study capitalized on these observations by prospectively analyzing a large collection of HNSCC cases for cyclin D1 expression by IHC and found that high levels served as an excellent predictive biomarker for selecting patients who would receive the largest possible benefit from cisplatin-based chemotherapy before surgery and postoperative radiotherapy [33]. In a related study

addressing response to cisplatin-based chemotherapy, SNPs in *XPF* (essential for DNA repair) were found to impact expression, and prospective analysis of HNSCC cases found that those cases expressing low levels of XPF correlated with more sensitivity to genotoxic agents such as cisplatin and radiation and overall predicted a better clinical outcome, with progression-free survival as the primary endpoint [129].

A mention should be made about EGFR, identified by [24], which is now known to be overexpressed in ~90 % of HNSCC and, together with its natural ligands (EGF, TGF-α), provide the essential requirements for autocrine growth-promoting mechanism, resulting in unregulated proliferation of tumor cells [123]. These observations provided the basis for developing EGFR-targeted therapeutic and predictive medicine [23, 84]. However, the clinical efficacy of the first-generation reversible EGFR small molecule inhibitors (gefitinib, erlotinib) has been limited, and the basis of this is unclear, especially as EGFR mutations are infrequent unlike in NSCLC and are found not to correlate with HNSCC therapeutic response [60]. Furthermore, the recent development of antibody-based biologics targeting the extracellular domain of the human EGFR has shown excellent promise for cancer therapy. These include a fully humanized (ABX-EGF) and chimeric mouse: human (Fv regions of a murine anti-EGFR antibody with human IgG1 heavy and kappa light chain constant regions [IMC-C225: cetuximab]) antibodies that bind EGFR with extremely high affinities (kd ~ 0.5 nM). The antibodies function by blocking the mitogenic and survival signaling pathways usually activated by EGFR, with the concurrent antibody-mediated receptor dimerization, receptor downregulation, and growth inhibition and tumor regression following [38, 47, 56]. Having gained FDA approval in 2006, cetuximab has shown mixed results for HNSCC, and conflicting reports as to its prognostic significance have been raised, largely due to the fact that only a small fraction of the patients (~10 %) on the treatment arm showed prolonged response, with the underlying basis of the remaining 90 % who fail to respond remaining unclear [131]. Several lines of investigation to understand predictive makers that might stratify HNSCC patients with already identified EGFR overexpression likely to benefit the most from receiving cetuximab are ongoing. Likely mechanisms leading to resistance to EGFR-targeted therapies include a mutation (S492R) in the ectodomain of the receptor, which essentially prevents the binding of cetuximab but not to the natural ligands and consequently leads to loss of efficacy of the interned therapy [88]. In another study to address this treatment failure, long-term cetuximab treatment was found to manifest strong activation of HER2, HER3, and cMET, which may potentially contribute to resistance by the EGFR binding and consequently activating HER2 or HER3 and maintaining the mitogenic Akt and MAPK signaling [133]. Resistance may also arise in principle due to the presence of activating mutations in key molecules (*PI3K* and *Ras*), which can independently stimulate EGFR downstream mitogenic signaling which essentially will negate any therapeutic value of EGFR-targeted therapies [7]. More recently, recent data suggest that TGF-β may be adding fuel to this process [8]. In this particular study, EGFR-targeted therapy in HNSCC was found to increase the levels of TGF-β which in turn activated the Akt-mediated survival signal and suppress the key effector

molecules involved in cytotoxic function [8]. Of interest, though, EGFR-targeted inhibitor-induced toxicity in the form of acneiform rashes manifesting as inflammatory papules and pustule in the skin areas of the face, neck, arms, scalp, and upper torso is now reported to be associated with better overall survival in several cancers including HNSCC, and overall these mechanism-based toxicities are an indicative biomarker of treatment efficacy [66]. Finally in this section, cell surface receptors on HNSCC may be exploited for ligand-directed and targeted delivery of cancer drugs to squamous tumors, in the same manner as described earlier for T-DM1 for breast cancer. By the virtue of the specific affinity of EGF for its cognate cell surface receptor, EGFR, recent work from our group has demonstrated that drug delivery bioconjugates consisting of attachment of low levels of cisplatin and EGF to single-walled nanotubes bioconjugates were much more efficient at killing EGFR expressing HNSCC cancer cells than untargeted controls containing the same drug compared, and these observations were consistent with receptor-mediated internalization of the EGF-attached bioconjugates into tumor cells and consequently induce tumor shrinkage [9]. This approach holds promise for targeted cancer drug delivery system, whereby cytotoxic drugs may be given at lower doses to minimize toxic side effects and help to localize the drugs effect to the tumor area. However, the use of nanoparticles in the clinics remains challenging [57]. As previously mentioned, biomarkers for HNSCC do not exist for routine clinical use, and while the aforementioned molecules represent promising candidates for fulfilling this shortfall, several studies of note analyzing HNSCC and salivary genomes (DNA and RNA) and proteomes have made remarkable advances on discovery and development of HNSCC relevant biomarkers as well as provided novel strategies for sensitive detection and measurement of these molecules for potential use in biomarker-driven personalized oncology, as discussed below.

6.4 HNSCC Genomic and Epigenetic-Derived Biomarkers

Understanding the complexities of the human cancer, genome now represents an excellent opportunity for the translation of this information into predictive and personalized medicine. Realization of this is now upon us, largely due to the ease of accessibility to cost-effective NGS, potentially allowing for crucial information to be transferred rapidly to patient care. Recent studies have now utilized this approach of large-scale sequencing to scan the HNSCC genomic landscape to essentially indentify alterations involved in cancer pathogenesis. DNA from 32 untreated HNSCC underwent analysis by this method, and a total of 609 somatic mutations were confirmed with an average of 19 alterations per tumor. For validation, the authors chose only those genes (or closely related) that were found mutated in at least 2 of the 32 tumors and sequence validated in an independent sample set. Surprisingly, alterations in only a small subset of genes of the original group (*TP53*, *NOTCH1*, *CDKN2A*, *PIK3CA*, *FBXW7*, *HRAS*) generally implicated in HNSCC were found in more than one tumor sample. The same study also reported a large proportion of the *NOTCH1* mutations were predicted to truncate the protein

product, indicating the gene product may be associated with tumor suppressive potential in HNSCC [1]. In a parallel study, whole-exome sequencing was performed on 74 HNSCC tumors, and of interest these were preselected for analysis based on risk factors (alcohol, tobacco, HPV), anatomical sites (oral cavity, oropharynx, hypopharynx, larynx, sinonasal cavity), and the presence of previously reported common (*CCND1*, *CDKN2A*) and less frequent (*MYC*, *EGFR*, *ERBB2*, *CCNE1*) alterations. From the many mutations detected, including those reported by Agrawal et al. ([1]), the study found alterations in genes (*NOTCH1*, *IRF6*, *TP63*) regulating squamous differentiation in 30 % of the tumors analyzed [124]. Collectively, both studies point to the existence of an enormous diversity in the genetic alterations leading to HNSCC development and progression.

Loss of heterozygosity (LOH) in key chromosomal loci as molecular markers of potentially malignant oral lesions at risk of developing full-blown carcinoma is raising interest, especially as there are no *bona fide* markers that can stratify potentially malignant lesions into those at low and high risk for malignant progression. Early studies have shown that a small fraction of benign oral squamous lesions harbor LOH at 3p14, 3p21, and/or 9p2l, and these may be associated with the early processes of tumorigenesis in HNSCC [78]. With this in mind, a risk model was proposed whereby allelic loss of these regions may predict malignant conversion of low-grade dysplasia compared to those with intact regions [110]. Using this concept and extending the LOH markers to other chromosomal regions, a prospective and population-based study consisting of 296 patients classified into low- and high-risk oral dysplasia groups was initiated. The study found that ~1 % of low-risk lesions (retention of 3p and 9p) progressed, and the high-risk cohort was prone to a ~22-fold increase in risk of malignant progression. Notably the inclusion of LOH markers on 4q and 17p helped to improve the risk prediction and likely set the stage for making informed decisions on treatment options [143].

Epigenetic alterations, reflected by changes to the 5-methylcytosine (methylation) content of genomic regions, are essential for normal cellular function and development, and aberrant methylation can give rise to cancer [53]. These altered epigenetic events including microRNAs (miRNAs) and promoter methylation occurring in HNSCC afford excellent opportunities for identifying target genes that may have predictive values. In this regard, promoter methylation of *miR-137* is now reported to be a frequent event in HNSCC, and a recent study found that this was essentially associated with an unfavorable outcome of HNSCC patients, likely due to involvement of one of the target genes, cdk6, in increased proliferative capacity [67]. The promoter sites (miR-9-1, miR-9-2, miR-9-3) of *miR-9* have also been investigated in a large HNSCC and control tissue sets, and methylation of miR-9-1 and miR-9-3 was higher in those samples than were oral and oropharyngeal carcinomas, with potential for these sites to be developed for diagnosis for this cancer subset [85]. In another study, a genome-wide array using the BeadChip (Illumina) was used to measure DNA methylation in HNSCC tissue samples and by way of a method for classifying CpG loci that allows functional sequence elements to dictate clustering essentially found 13 CpG loci, characterized by polycomb group target genes, mammalian interspersed repeats, and transcription factor binding sites, to be

associated with poor patient survival, with *TAP1* and *ALDH3A1* genes the most highly implicated with this strategy [102]. Changes in salivary miRNAs and epigenetic changes occurring in cells isolated from salivary rinses are discussed in a later section.

Notwithstanding, there is still a perceived forward inertia to integrate whole-genome sequencing into health-care systems, essentially to indentify defective genes in tumors that may offer the most effective treatment to newly diagnosed cancer patients and for research scientists to develop newer therapies. However, such efforts need careful coordination from a multidisciplinary team with expertise in clinical oncology, genomics, bioinformatics, pathology, bioethics, and genetics. This will ensure integration of whole-genome and whole-exome capture, RNA sequencing, and the analysis of the data to identify aberrations that can be translated to patient care in a timely fashion. It follows that this approach can be applied to patients with HNSCC providing a unique opportunity to offer personalized treatment and for the development of additional targeted therapies [111].

6.5 HNSCC Proteomics-Derived Biomarkers

Although two-dimensional electrophoresis (2DE) has been used extensively to detect and characterize proteins present in cancer samples, several highly sensitive platforms now offer steam-lined alternatives to identify, measure, and catalog proteins [31]. This is now more evident as large-scale initiatives using mass spectrometry (MS) are now in place to essentially search and characterize all human protein-coding gene products, including low abundant proteins not detected in previous studies [64, 94, 95]. Notably, an estimated 25 % of the human proteome still remains uncharacterized, largely because of the large dynamic range of proteins in complex samples, those in low-abundance and possibly associated with disease, usually end up falling below the detection limit. Thus, this portion of the uncharacterized proteome now affords a unique opportunity for discovering additional protein biomarker associated with HNSCC [79].

Previous efforts for biomarker discovery include matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry (IMS), which allows for the systematic analysis of both low (~1 kDa) and high (~100 kDa) molecular mass proteins across complex tissues, and the direct comparison to histopathological features [115]. The fact that this technique affords the simultaneous analysis of hundreds of proteins without the need for complex labeling or sample preparation, it is well suited for cancer biomarker discovery, albeit with limited application to HNSCC [97]. Similarly, with the use of MS and improved protocols for sample preparation in recent years, advancements have been made for the quest for biomarker discovery [136]. As one approach, mass spectrometric identification of proteins secreted from cancer cells may aid in the identification of biomarkers that may not only help to better understand the complexities of tumor behavior and its environment but also for their clinical utility. In one such study, direct MS-based proteomic profiling was performed on the secretome of HNSCC, and after

cross-validating the data, high levels of PLAU, IGFBP7, MMP14, and THBS1 were essentially associated with a poor outcome [116]. However, using a different form of analysis secreted levels of α-enolase, PPIA, hnRNPK, and some members of the14-3-3 family of proteins have been reported to be upregulated in HNSCC [106], and while the methods used may account for the differences seen between the two studies, combining the information to form a larger panel of protein markers may help to increase the predictive power to correctly detect cancer. Noteworthy, the aforementioned markers are likely not to be HNSCC cancer specific, as many of these proteins have been reported to be elevated in several other cancers [80]. On the other hand, the rational use of mass spectrometry may also lead to the identification of much needed protein kinases that may have value as key therapeutic targets for HNSCC. Using a chemical proteomics approach, consisting of a mixed kinase inhibitor matrix (kinobeads), which can potentially bind to a large portion of the expressed kinome and related proteins and after, these may be analyzed by intensity-based label-free quantitative MS [6]. Building on this innovative capability, 34 HNSCC lines were analyzed and identified 146 relevant kinases with some showing marked differences in expression, and after loss of function validation, the study found that 9 of these targets (AURKA, EPHA2, EPHB2, EPHB4, JAK1, LYN, NEK9, RIPK2, WEE1) have a direct impact on promoting and maintaining cell survival and possibly representing novel targets [137].

Recently, laser capture microdissection (LCM) integrated to MS has been gaining acceptance for discovery-based identification of cancer-related molecules within the cellular heterogeneity of the tumor mass. Using this concept and utilizing our expertise in LCM combined with improved buffers and separation techniques, work from our group implemented a minimal workflow that combined LCM with liquid chromatography–tandem mass spectrometry (LC–MS/MS) and successfully identified, in a small cohort, freshly frozen sample set proteins expressed specifically in histologically normal oral squamous epithelium and matching cancer compartment [5]. This methodology resulted in the identification of several differentially expressed proteins; among these keratin 13 and Hsp90 after validation were found to be reduced and abundant in tumors, respectively. However, freshly frozen and clinically annotated samples in certain settings may not be readily available, and instead formalin-fixed paraffin-embedded (FFPE) pathological cases which are clinically annotated and banked can offer an alternative source of material for such LCM- and MS-based proteomic studies. Furthermore, while FFPE samples allow for a more precised LCM based on the excellent histological preservation, the proteins within the tissue architecture get chemically cross-linked, making extraction challenging. Notwithstanding, recent work from our group combined LCM with recently developed techniques for protein extraction from FFPE tissues and a novel proteomics platform that consisted of a nanoflow reversed-phase liquid chromatography followed by linear ion trap MS analysis [98]. From ~20,000 LCM-enriched cells from FFPE tissue sections of normal oral epithelium and well (WD), moderately (MD), and poorly (PD) differentiated HNSCC cases, several hundred proteins were identified from each sample set. Using peptide counts as an approach for monitoring abundance of the proteins, the peptide distribution of the cytokeratins 14, 17,

and 16 was found to be lower in the normal oral mucosa compared with the WD, MD, and PD tumor samples. By contrast, the peptide distribution of cytokeratin 4 was higher in normal (~77 %) compared with the tumor samples (~4–12 %). Other molecules of interest from the analysis that may have potential as biomarker were those involved in cell-to-cell interaction (desmoplakin, democollin 2A/2B, desmoglein 3 precursor, plakophilin 1, and plakophilin 3). As mentioned earlier, these observations led us to further validate desmoglein 3 (DSG3) for its ability to correctly predict HNSCC progression in newly diagnosed patients [100]. Other proteins of interest from this FFPE proteomic analysis included HSP27 and HSP70, vimentin, glutathione S-transferase, and integrin β4, where the distribution of peptides was found to be essentially low to undetectable in normal samples when compared to different tumors. The workflow that our group optimized can be elaborated under the premise that it may lead to in-depth biomarker discovery. One study utilized O labeling of digested proteins extracted from ~10^6 LCM-enriched cells from three pairs of matched normal and tumor and 2D-LC separation with MALDI-TOF/TOF, as a method of choice for analysis, identified and quantitated ~977 proteins where 53 were deemed to be upregulated while 27 were downregulated. Notably, validation found that some of the upregulated molecules were members of the type I IFN signaling pathway and likely impact the proliferative potential of the tumor cells [21]. Finally in this section, building on our expertise in LCM and the ability to print various antibody array formats essentially constituting a reverse Western blot approach, we questioned if these two techniques could be combined to search for known proteins that could be involved in cancer progression. Thus, we used over 300 available antibodies against proteins involved in cancer, for example, cell proliferation and survival, to array onto nitrocellulose-coated glass slides and after hybridized with biotinylated LCM-procured protein extracts from cases of HNSCC and matching normal mucosa, to assess the nature of the proteins that may be involved in cancer progression [59]. Notably, levels of INF-α, Rsk, and RAR-α were seen to increase in protein extracts from stromal boundary adjacent to the tumor and can be likely used for identifying tumor margins. Overall, these case studies illustrate the vast amount of valuable information regarding the still unknown molecular mechanisms promoting the malignant conversion of the normal oral epithelium, from which suitable biomarkers of disease development and progression can be identified and novel therapeutic targets for HNSCC can be gained.

6.6 HNSCC Salivary and Blood-Derived Biomarkers

With ease of access and noninvasive manner of collection, saliva holds excellent potential for biomarker discovery [104]. Indeed, both the salivary genome and proteome have been extensively investigated using widely available high-throughput methods aimed at identifying molecules that may reflect cancer-related changes in the saliva for potential use for early diagnosis and prognosis of HNSCC and/or postoperative monitoring for recurrences and treatment efficacy. However, human saliva is a biological fluid and prone to degradation if collection, processing, and

storage conditions are not thoroughly optimized and validated. Recent data has determined that by adding 20 % ethanol to saliva samples, the salivary proteome became stabilized and remains viable for up to 2 weeks at room temperature, allowing sufficient time for transportation and storage, prior for use for biomarker discovery and biomarker validation [138]. For analysis of genomic DNA from saliva collection, a caveat for consideration is that a large fraction is likely to be from host immune cells and bacteria [81], and stringent QC should be implemented in order to ensure that sufficient quality and quantity of human DNA is extracted.

Nonetheless, with strict criteria for collection and storage, saliva is now becoming used routinely for single biomarker studies. As CD44, a transmembrane hyaluronan-binding glycoprotein, is overexpressed in HNSCC lesions compared to other solid tumors, a recent study investigated whether levels of salivary-soluble CD44 might have diagnostic value. Saliva samples from HNSCC patients and normal controls were analyzed for soluble CD44 by enzyme-linked immunosorbent assay (ELISA), and levels were found to be significantly elevated in cancer samples compared to controls, irrespective of stage, treatment, or size, and hence, it can offer an effective marker for early detection of HNSCC [39, 105]. Similarly, saliva from HNSCC patients and normal controls was subjected to a proteomics analysis using 2DE and MALDI-TOF MS and found that transferrin levels were higher in cancer patients, and a transferrin-based ELISA predicted 100 % for patients with early-stage HNSCC [54].

As previously mentioned, a panel consisting of 4–10 carefully selected markers is preferred for increasing the accuracy of the predictive power, and in light of this, a recent study analyzed eight cancer biomarkers in saliva samples from patients with overt tongue carcinomas ranging from early to advance stages of the disease. When simultaneously measured by ELISA, significant changes in levels of the protein were determined, but the sensitivity and specificity nevertheless were in a broad range of 40–100 % [117]. Another consideration that is crucial to move from biomarker panels in the exploratory phase to clinical application is the adequate cross-validation of biomarker assays including method optimization. A recent study was specifically designed to validate whether seven mRNAs (IL-8, SAT, IL-1B, OAZ1, H3F3A, DUSP, S100P) and three proteins markers (IL-8, IL-1B, M2BP) previously reported as potential biomarkers for oral cancer detection were able to stratify untreated patients with differing stages of HNSCC (I–IV) from healthy subjects in five population-based, case–control studies totaling 395 subjects. The study found that the maker levels did not correlate with tumor expression but were essentially elevated in cancer saliva compared to control and with IL-8, IL-1B, and SAT determined as top performers across the different cohorts [30]. Lessons learned include the need for thorough cross-validation to ensure the sturdiness of the oral cancer salivary biomarkers, including the need for standardized technical methodologies and improvement in inter-operator and inter-sample variations [30].

Due to the nature of their structure, miRNAs can remain stable in body fluids, making this class of molecules ideal for exploiting as saliva biomarker of HNSCC. Using this strategy, a recent study found that from screening ~300 miRNA in RNA samples by RT-preamplification–qPCR isolated from saliva from HNSCC

patients and matched controls, two (*miR-125a* and *miR-200a*) were significantly lower in HNSCC, potentially offering an noninvasive and rapid diagnostic method for the diagnosis of HNSCC [96]. Very similarly, targeted analysis of a few miRNAs can yield invaluable observation, and in this regard, the analysis of *miR-31* in saliva samples found that its levels are largely elevated in HNSCC irrespective of stage, which interestingly were reduced after surgical excision, suggesting that *miR-31* may serve as a predictive biomarker for early detection and treatment response [72].

Salivary rinses are well suited for molecular screening of potential epigenetic changes of key genes in high-risk HNSCC patient group. A panel of promoter hypermethylation marker genes (*p16*, *CCNA1*, *DCC*, *TIMP3*, *MGMT*, *DAPK*, *MINT31*), identified from prior studies to be predictive of local recurrence and overall survival, were put through a rigorous validation in a large independent patient cohort [19]. In this study, salivary rinses were prospectively collected from 197 patients with previously untreated HNSCC, and DNA extracted from the cellular material collected from the rinses underwent methylation status of the gene promoters by quantitative methylation-specific PCR. Overall, close to 47 % of the samples exhibited promoter hypermethylation of at least 1 of the 7 genes and pertinently found promoter hypermethylation of TIMP3 to be an independent prognostic factor of local recurrence and overall survival, allowing for close monitoring of patients at risk for recurrences [125]. Using a similar strategy, the aberrant promoter hypermethylation of *KIF1A* and *EDNRB* was associated with the presence of oral cancer and premalignancy, therefore representing potentially useful markers for risk assessment of dysplastic or clinically low-risk lesions that would have otherwise not met the clinical criteria for biopsy [101].

Blood-derived biomarkers have potential for cancer detection and disease progression, but very few have been described for HNSCC. In this regard, plasma levels of MMPs and their inhibitors have been investigated for their predictive value, and in a study involving screening of 136 patient samples by ELISA, low levels of TIMP-1 (<7 nM) notably correlated with a favorable survival compared to elevated levels >11 nM, while MMP-8 levels had no impact on patient survival [103].

Finally, promising HNSCC biomarkers need to undergo extensive validation before their full potential can be realized, but unfortunately it is at this stage where most molecules fail. As an example, osteopontin (OPN), which is known to be associated with advanced HNSCC, is inversely correlated with Von Hippel–Lindau (VHL) protein levels, which has a direct impact on tumor oxygenation and hypoxia [68]. In this setting, OPN has all the requirements for use as a dual prognostic and predictive biomarker for HNSCC patient selection for hypoxia-targeted therapy. To test this, a large multicentered phase III prospective study was conducted to assess the prognostic and predictive significance of OPN in advanced HNSCC, but the study essentially found no evidence that high plasma OPN levels were predictive of benefit from treatment using hypoxic cell cytotoxin [71]. While the time lost together with the financial burden would be unjustifiable, these would have been without doubt even higher if biomarkers had otherwise readily passed to the clinic with less stringent criteria, reiterating the need for strict validation procedures involving larger sample sets and in multicenter studies.

6.7 Strategies for HNSCC Biomarker Detection

Currently, the main challenges for accurate detection and measurement of cancer biomarkers are their low abundance in tissues and body fluids, narrow margin between normal and elevated levels, and the insufficient sensitivity of most assays used [113]. Thus, the single most important goal of a cancer biomarker is knowing the presence of the smallest number of tumor cells before further growth, when clinical outcome and prognosis are still favorable [50, 113]. A number of techniques for multiple biomarker protein measurements have been developed in the past. Most of these methods utilize immunoassays for the detection of proteins, essentially based on specific binding of an antibody to its target. ELISA is the gold standard for clinical protein measurements with low sensitive detection limits (DL), but limitations include prolonged assay time, sample size, equipment cost, and simultaneous measurement of multiple proteins. Current multiplexed methods for proteins include polynucleotide barcodes; optical detection on antibody microarrays and multiplexed bead platforms have excellent DL (picomolar range) but in general require considerable technical expertise and relatively high costs. To this end, the implementation of accurate biomarker detection in clinical settings requires rapid, technically reliable, and inexpensive devices to measure multiple proteins in patient samples, but these biomarker-based methodologies are not generally available in the clinic [76, 113]. Therefore, the pursuit of new, rapid, advanced, reproducible, and cost-effective diagnostic devices for ultrasensitive multiplexed measurement of biomarker proteins for cancer, using different aspects of nanotechnology and novel labeling techniques in conjunction with electrochemical detection for improved accuracy, is now gaining acceptance [34, 58, 113].

Self-contained, enzyme-linked electrochemical immunosensors using nanostructured electrodes layered with gold nanoparticles (AuNP) featuring antibodies attached to the sensor surface and paramagnetic beads (MB) labeled with horseradish peroxidase (HRP) and secondary antibody for signal amplification and high sensitivity, capable of detecting multiple proteins at 5–50 fg mL^{-1} (attomolar) levels, have paved the way for measurement of proteins. In this regard, work from our team has led to the development of an 8-sensor AuNP array contained in a microfluidic channel interfaced with a syringe pump and sample injector. Antibodies to different protein biomarkers were attached to the 8 sensor elements and paramagnetic beads (MB) loaded with 400,000 HRPs and thousands of secondary antibodies to capture analyte proteins from the sample separately (off-line) to minimize nonspecific binding. This allows the washed MB with captured proteins to be injected into the microfluidic array and incubated with the 8 sensors such that sensor antibodies bind their specific protein partners attached to MB and elicit an amperometric signal, requiring ~5 μL of sample and ~50 min. Using this setup, immunosensors were used to validate a 4-cancer biomarker protein panel (IL-6, IL-8, VEGF, VEGF-C) by analyzing a cohort of 136 serum samples from HNSCC patients and cancer-free controls. Statistical analysis indicated clinical specificity of 98 % and sensitivity of 89 % for oral cancer detection based on normalized means of the 4-protein assays, highlighting the importance of multiplex assays of

biomarkers for accurate diagnosis cancer, specifically those that are asymptomatic and at early stage when prognosis is still favorable [76]. Figure 6.3 shows a schematic representing 1-sensor surface layered with AuNPs and attached antibodies of interest (left), MB with HRPs and secondary antibodies are used to capture the protein of interest from complex human serum (or tissue extracts) separately (middle), and after the washed, slurry is applied through a microfluidic channel to the sensor (right).

Electrochemical sensors have also been developed for the multiplex and concurrent detection of biomarkers under the premise of achieving a favorable diagnosis compared with a single biomarker [112]. In a recent study, a 16-array gold electrode chip, precoated with fluorescein dual-labeled IL-8 mRNA hairpin probe or biotinylated human IL-8 monoclonal antibody separately, was used for the rapid (~10 min) screening of oral cancer and control of saliva samples for salivary IL-8 and found that combined levels of mRNA and protein under challenging conditions were essentially higher in cancer, giving a better area under the curve (AUC) compared to a single biomarker, allowing for increased accuracy in diagnosis of oral cancer in using a saliva-based screen [132]. Optical protein sensors and surface plasmon resonance (SPR) have also been optimized for detecting IL-8 as a single protein marker in saliva samples with the view of discriminating healthy patients from those with HNSCC [126, 139].

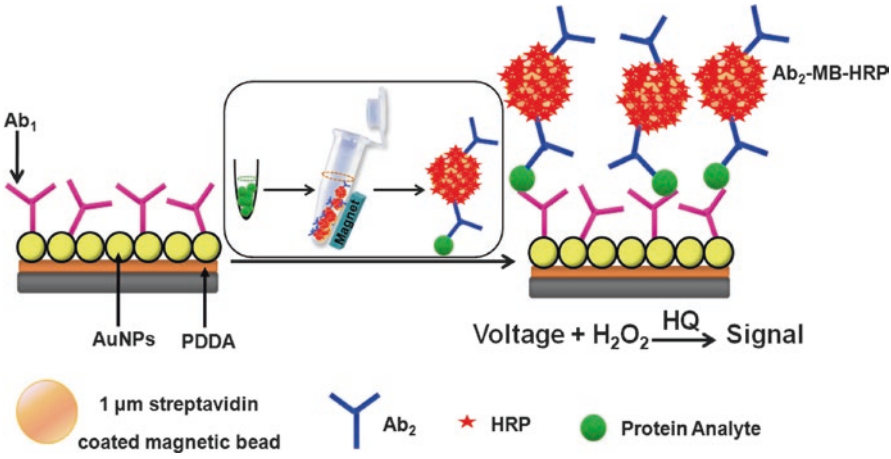

Fig. 6.3 Scheme depicting the ultrasensitive detection of proteins in complex serum and tissue matrix. A single sensor (from the eight-sensor format) layered with AuNPs covered with capture primary antibodies is shown. Next, protein of interest in complex serum or tissue extracts is captured separately on Ab_2-MB-HRP bioconjugates consisting of ~400,000 HRP labels and 100,000 AB_2 (secondary antibody). This is followed by the Ab_2-MB-HRP complex being magnetically separated, washed, and injected into the eight-sensor immunoarray, which is connected to a pump and injector valve (not shown). Amperometric signals are then developed by injecting a mixture of H_2O_2 and hydroquinone, and the peak output is used for generating calibration plots which are used to measure protein levels in biological samples. See further details in Malhotra et al. [76]

6.8 Future Perspectives on Cancer Biomarkers and Oncology Drug Development

Key consideration that needs to be embraced prior to using validated biomarkers in cancer therapies is that from an estimated 90 % of drugs currently on the market work in ~40 % of patients, and while current technological platforms have excellent potential to generate biomarker targets, the vast majority with a limited knowledge of their biological function in fact fail to make an immediate impact on predicting whether new therapies are working on cancer patients [28]. One likely reason for this may point to a less-than-optimal dissemination of data from biomarker-driven oncology drug development clinical trials involving tumor biopsies. This is likely stemming from poor patient accrual, poor quality samples, lack of appropriate assays, poorly designed studies, and an unclear interpretation of the data, clearly indicating the need for scientifically robust and validated biomarkers and their use in well-designed clinical trials [40]. In this context, two exemplary clinical trials are noteworthy (BATTLE [A biomarker-Integrated Targeted Therapy Study For Lung Cancer Elimination] and I-SPY2 [Investigation of Serial Studies to Predict Your Therapeutic Response With Imaging and molecular Analysis]). These trials were designed around predefined biomarkers and their underlying biological understanding and have helped to impact treatment, for example, by identifying drug candidates that are ineffective and those deemed to confer excellent efficacy which can be prioritized for fast regulatory approval [32]. Conversely, the lack of suitable biomarkers still plagues the favorable conclusion of late-stage clinical trials. For example, cancers that are fuelled primarily by IGF and the insulin-like growth factor 1 (IGF1) receptor (IGF1R) pathway offer an excellent therapeutic opportunity [141]. Indeed, several IGF1R-targeting antibodies in late stage development have all but failed to gain final approval, largely due to the lack of predictive biomarkers to identify those patients that are likely to benefit the most from the therapy [27]. The molecular basis of this may stem from the fact that both IGF1 and IGF2 activate the insulin receptor in addition to IGF1R, and the expression levels of these molecules do not confer a predictive value for efficacy with IGF1R-targeted biologics [141].

Due to the complexity and heterogeneous nature of cancer as a disease, improved assay systems, such as those utilizing isogenic cells lines that accurately model the disease causing genetic alterations of cancer patients, are well suited for validation and could help to improve the predictive potential of cancer biomarkers for drugs targeted specifically to a more responsive patient population [61]. Similarly, suitable animal models such as those carrying patient-derived tumor xenografts also offer value for validating predictive biomarkers and allowing for the selection of the best treatment [48]. Furthermore, gene targeting efforts using *zinc finger* and *TALEN effector* nucleases, for engineering mice recapitulating genetically and biologically human diseases, can lead to much better models of human cancers, thus offering faster and more flexible approaches for biomarker validation [89]. For example, using *TALENS* which are essentially engineered DNA nucleases consisting of two domains, a custom-designed DNA-binding domain that attaches to the chosen site in the genome and a nonspecific nuclease domain for the cutting. Thus, by binding

the *TALEN* nuclease at either side of the target gene, double-strand breaks are made, and after DNA repair rejoining the cut ends of DNA, the target gene is effectively manipulated [89]. In this manner, any genetic alteration implicated in cancer can be biologically validated and assessed for therapeutic validity. Finally, validation of cancer biomarkers is being made easier largely due to Human Proteome Organization-endorsed Human Protein Atlas and Human Antibody Initiatives, to effectively generate a protein atlas for the expression and localization of human proteins in normal and disease tissue and validated antibodies from many different sources and help to better understand the protein targets of interest [13, 15, 79]. Overall, we can expect that the emerging information from large multi-institutional and international initiatives, combined with the wealth of information regarding the human HNSCC proteome, oncogenome, and signalome, will soon result in the identification and validation of suitable biomarkers for the early detection of HNSCC premalignant lesions as well as markers predicting a beneficial clinical response to newly developed molecular-targeted therapeutic options to prevent and treat HNSCC.

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Retracted: Oral Potentially Malignant Disorders

7

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The World Health Organization (WHO) workshop in 2005 redefined all oral lesions with a potential for a malignant transformation to be grouped under the title "potentially malignant disorders." The traditional terminologies of premalignant lesions and premalignant conditions have been abandoned. There are several lesions that fall under this title; however we will limit our discussion to the most commonly seen lesions in clinical practice; these include lichen planus, leukoplakia, erythroplakia, erythroleukoplakia, erythroplakia with ulceration, proliferative verrucous leukoplakia, and submucous fibrosis. All the aforementioned lesions are predominantly found in the oral cavity. Potentially malignant lesions of the skin can also be found in the head and neck and however are beyond the scope of this chapter and will not be discussed.

7.1 Oral Lichen Planus

Oral lichen planus (OLP) is a complex, chronic, inflammatory disease that affects about 1–2 % of the population. Lesions can occur both in the oral cavity and on cutaneous tissue. The reported malignant transformation of OLP is less than 1 %. Other studies have demonstrated that the rate of transformation ranges from 0.4 to 5.2 % [1, 2]. The etiology of OLP is unclear currently; however there is some evidence to suggest a T-cell-mediated process [3, 4]. The tissue in such

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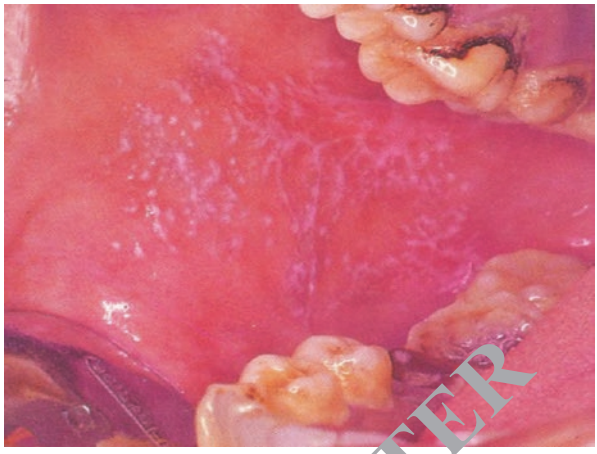

Fig. 7.1 Oral lichen planus on the buccal mucosa showing the classic reticular pattern

lesions shows a local activated T-cell population with increased expression of cytokines and altered adhesion molecule expression [5]. In addition, therapies that suppress T-cell-mediated immune responses can cause clinical improvement of OLP [6].

Clinically, OLP presents in a variable manner ranging from a keratosis to erythroleukoplakia. Ulceration or erosion of the mucosa with associated cutaneous manifestations can occur [7, 8]. Typically inflamed lesions and ulcerative lesions are more painful and cause a burning sensation compared to classically described white lacy reticular pattern seen on the buccal mucosa (Fig. 7.1). The four distinct clinical patterns most described are reticular, erosive, plaque-like, and bullous [9]. The reticular type is most commonly seen as a lacy network on the buccal mucosa and often referred to as Wickham striae. Erosive lichen planus appears as ulceration with a whitish-yellow pseudomembrane [10]. The typical whitish raised lesion that cannot be rubbed off the tongue is typically the plaque-like lichen planus. Bullous lichen planus is a very rare form and characterized by large bullae most commonly seen in the posterior buccal mucosa.

Management of OLP starts with the confirmation of the pathology by biopsy. A thorough history is important in understanding disease progression and the treatment is dependent on the symptomatology. Symptomatic relief is often the first-line treatment with immunomodulation with topical steroids. Persistent painful erosive lesions that are not responsive to topical steroid therapy are usually treated with a short course of systemic steroids [7].

7.2 Leukoplakia

Leukoplakia is defined as a whitish patch or plaque that cannot be characterized clinically or pathologically as any other disease and is not associated with any physical or chemical causative agent, except the use of tobacco as described by the World

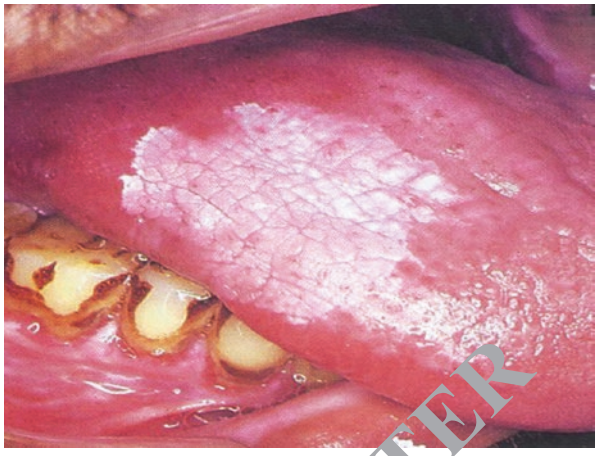

Fig. 7.2 Homogeneous leukoplakia of tongue

Table 7.1 Differential diagnosis of oral leukoplakia

Lesion	Diagnostic features
Candidiasis	The lesion appears as curdy white symmetrical lesion of oral cavity that can be wiped away leaving bleeding mucosal bed. The diagnosis can be confirmed by identifying candida hyphae in the biopsy
Frictional keratosis	It occurs because of constant mechanical irritation from foreign body or sharp tooth. Resolution of the lesion after removing the offending irritant is diagnostic of the lesion
Hairy leukoplakia	It is resultant from elongated filiform papillae of tongue giving a white furry appearance to the dorsal surface of tongue
Fordyce granules	These are ectopic sebaceous glands often found on the buccal mucosa. The small punctate white lesion located on the buccal mucosa is suggestive of these lesions
Leukoderma	Symmetrical pale white lesion of the buccal mucosa
Linea alba	Linear white line corresponding to the occlusal plane
White sponge nevus	It is characterized by formation of thickened, velvety sponge-like lesions often found on the buccal mucosa. Mutations in KRT4 or KRT13 have been implicated in this condition. There may be family history of similar lesions. It can be present from birth
Nicotine stomatitis	It is also called smoker's palate. It typically appears as white patch of the palate with red dots representing minor salivary gland duct openings
Chemical burn	Local application of medication such as aspirin or hot food can leave white mucosal patch

Health Organization in 1984. Recently, the WHO (2005) changed the definition of leukoplakia as "a white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer" [11, 12] (Fig. 7.2). Leukoplakia primarily being a clinical diagnosis, it needs to be distinguished from other clinically similar lesions. The list of conditions is given in Table 7.1.

The prevalence of leukoplakia worldwide is approximately 2 %; however the range is from 0.2 to 3.6 % [13–15].

The main etiological agent of leukoplakia is tobacco and there is a strong evidence to demonstrate this relationship [16, 17]. Alcohol is also considered to be a risk factor in the development of leukoplakia, independently and synergistically with tobacco [18]. In 1980, the WHO classified leukoplakias into homogeneous and nonhomogeneous groups. The homogeneous group clinically describing a flat, thin, and uniformly white lesion was further divided into three lesions, namely smooth, furrowed (fissured), and ulcerated. The nonhomogeneous leukoplakia group was described as a white-and-red lesion, which is irregular or nodular. Another way of classifying leukoplakias was based on histologic basis of dysplastic vs nondysplastic leukoplakia. The 2005 WHO classification identified five separate histologic stages in the epithelial precursor lesions. Mild, moderate, and severe dysplasia and carcinoma in situ are used to describe these lesions and are often difficult to distinguish clinically. Dysplastic lesions are graded by severity as mild, moderate, or severe dysplasia or carcinoma in situ. Carcinogenesis is neither linear nor predictable, and it may occur over a variable time period; sometimes malignant transformation can take decades [19, 20].

In oral epithelial tissues, accumulating mutations (i.e., genetic progression), chromosomal damage and loss of cellular control functions are observed during the course of sequential histologic changes, which culminate in oral cancer [19, 20]. These changes are manifested as the transition from normal histology to early intraepithelial dysplasia and pre-neoplasia, through increasingly severe intraepithelial neoplasia to superficial cancer and finally invasive disease [19, 20]. Although the carcinogenic process can be relatively aggressive (e.g., in the presence of a DNA repair – deficient genotype or viral transformant such as human papillomavirus), these changes generally occur over a long time period [19–21].

Carcinogenesis is characterized by progressive loss of proliferation and apoptosis controls and increasing cellular disorganization, aneusomy (DNA content), and heterogeneity [20, 22, 23]. The appearance of specific molecular and more general genotypic damage is associated with increasingly severe dysplastic phenotypes. In many cases, crucial early steps include inactivation of tumor suppressor genes (e.g., mutation of p53 gene) and/or activation of oncogenes (e.g., *ras*). Carcinogenesis may follow multiple paths and be multifocal; not all cancers in a given tissue nor all cells in a given cancer may ultimately contain the same lesions. Progression to cancer may also be influenced by factors specific to the host's tissue environment.

Field cancerization refers to the development of tumors at multiple sites in the oral cavity. Slaughter et al. described this on evaluation of dysplastic epithelium adjacent to invasive tumors in head and neck cancers, leading to an increased development of second primary tumors [24–26]. The tumors were initially thought to develop from independent clonal cells; however, other studies have also shown that these clones would in fact be related to the primary tumors [25, 26]. These multifocal clonal cell nests may be a consequence of prolonged exposure to carcinogens making it very challenging to treat squamous cell carcinomas of the head and neck.

An insight into carcinogenesis highlighting progression from the earliest cellular changes through to carcinoma in terms of histopathology and genomics may provide the clinician a deeper understanding of the aforementioned clinical signs and

symptoms. Current scientific consensus is that this oral carcinogenesis occurs in a stepwise fashion hallmarked by an accumulation of genetic mutations sufficient for malignant transformation. Such genetic mutations predict changes in the normal maturation of keratinocytes, by affecting the control of the cell cycle, apoptosis, and terminal differentiation [19–21].

Such changes are manifested in the epithelial architecture as a transition from normal stratified squamous histology to epithelial dysplasia and then to invasive squamous cell carcinoma. The term epithelial dysplasia is a histopathological diagnosis rendered when cells with atypical morphology are detected within the epithelium.

Management of leukoplakia usually begins with removal of the source of the lesion. Tobacco cessation program enrollment with or without medication management is generally attempted for 2–4 weeks to look for reversal of mucosal changes. If no changes are noted in the lesion, then a definitive diagnosis should be established with a biopsy of the lesion. Biopsy of the lesion usually classifies leukoplakia into dysplasia or non-dysplastic leukoplakia. Non-dysplastic lesions can usually be followed every 6 months and mildly dysplastic lesions are typically managed in a similar manner. Moderate to severe dysplastic lesions are usually managed with complete excision or laser ablation to facilitate further histologic typing of the lesion. Excision of leukoplakia is however notoriously unpredictable with variable recurrence rates ranging from 0 to 30 % according to the literature [27–29]. Once excised, lesions are typically monitored closely with risk factor avoidance every 4–6 months.

7.3 Erythroplakia

An erythroplakia is a red lesion that cannot be classified as another entity (Fig. 7.3). Far less common than leukoplakia, erythroplakia has a much greater probability (91 %) of showing signs of dysplasia or malignancy at the time of diagnosis [30]. Such lesions have a flat, macular, velvety appearance and may be speckled with white spots representing foci of keratosis. Prevalence of erythroplakia is estimated at 0.02–0.83 % from the only studies from Asia [31].

Erythroplakia lesions usually present as solitary lesions unlike erosive lichen planus and erythematous candidiasis, which almost always occur in a bilateral and symmetrical manner. Lesions with an erythematous or red component (erythroleukoplakia) are far more likely to undergo dysplastic or malignant epithelial changes than leukoplakia [32–34]. Red lesions without a white component may also represent dysplasia, carcinoma in situ, or carcinoma and therefore such lesions must be carefully evaluated and preferably completely excised. Carcinogenic progression in patients with erythroleukoplakia has been shown to be almost fourfold that of the patients with homogeneous leukoplakia [34, 35]. Therefore, all patients with chronic white-and-red lesions, whether treated or not, should have periodic diagnostic biopsies in particular when clinical appearance changes or with the onset of new symptoms. It is particularly useful in patients with high-risk lesions to obtain detailed descriptive and photographic information of the lesions in order to optimize surveillance protocols. In a representative study of 257 patients, 58 % of the patients with

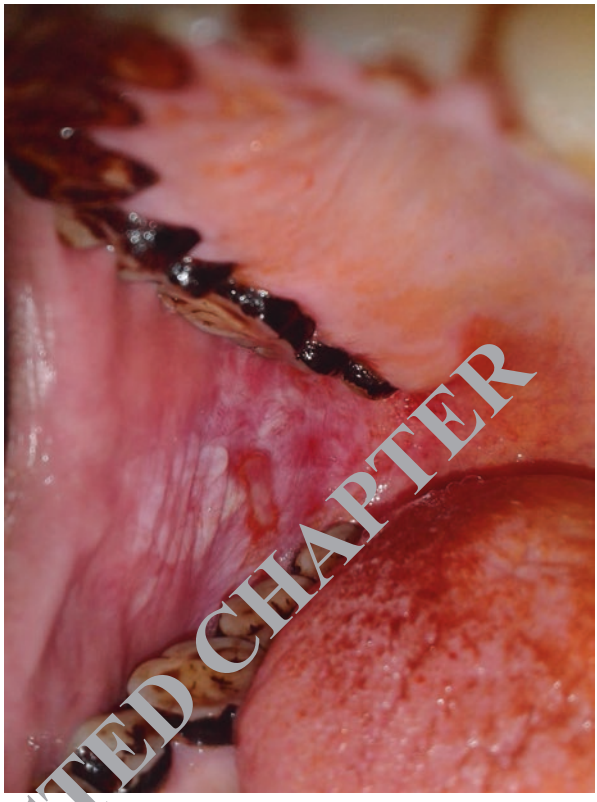

Fig. 7.3 Erythroplakia of buccal mucosa

leukoplakia had an associated erythematous area, whereas 84 % of the patients who eventually developed a carcinoma demonstrated a red component. Other studies have confirmed this association [33–35]. In Mashberg and Samit's prospective study of 222 asymptomatic oral carcinomas, 28 % were red only; 62 % were red and white; 97 % occurred in the mouth floor, oral tongue, and oropharynx; and 84 % were less than 2 cm at their largest diameter [34, 36].

Treatment of erythroplakia is usually by excision or by laser ablation. Unfortunately, there is no data about the recurrence rate after excision of erythroplakias. Despite this, it is prudent to continue to follow patients periodically to ensure no recurrence of erythroplakia or conversion to carcinoma.

7.4 Erythroplakia with Ulceration

Another rare but high-risk premalignant lesion is the chronic erythematous change associated with constantly recurring erosive changes. These lesions are often mistaken for "recurrent aphthous stomatitis of the herpetiform variety" or "nonspecific inflammatory immunopathologic vesiculoerosive disease."

7.5 Proliferative Verrucous Leukoplakia

Silverman and colleagues first described a unique form of leukoplakia found in 30 patients [37]. This group of lesions has a high risk of malignant transformation. The name "proliferative verrucous leukoplakia" (PVL) is due to the characteristic appearance – an expanding, exophytic/fissured white lesion (Fig. 7.4). PVL is a very high-risk precancerous lesion with high transformation and mortality rates, older (>60 years old) women outnumber men, less than one-third of PVL patients smoke, and there is usually multisite oral involvement [37].

The link between proliferative verrucous leukoplakia and oral cancer was first established by Hansen et al. in 1985 [38, 39]. Recently, the role of HPV as a causative factor for squamous cell carcinoma has been well established. The mechanism of HPV carcinogenesis is thought to be modulated via the HPV 16, 18, and 31 subtypes and can be found in premalignant and malignant lesions in the oral cavity [40, 41]. The molecular mechanism of HPV oncogenesis after infection is the disruption of the E6/7 viral oncoproteins. The E6 protein binds to p53 and promotes its degradation, while the E7 protein binds to the retinoblastoma protein. The two processes together lead to cell cycle dysregulation and malignant [41].

Two types of genital tract HPV in particular, HPV 16 and HPV 18, are genital viruses and known to cause the vast majority of cervical cancers, and one of them, HPV16, is also linked to oral cancer as well. These types of the human papilloma virus have E6 and E7 proteins with very strong binding capabilities. This allows HPV 16 to reproduce quickly and in great numbers, leading to uncontrolled reproduction of viral cells and eventually cancer. In the oral environment, HPV16 manifests itself primarily in the posterior regions such as the base of the tongue, the oropharynx, the tonsils, and the tonsillar pillars. These oncogenic or cancer-causing versions of HPV are also responsible for other squamous cell carcinomas, particularly of the anus and penis.

In general it appears that HPV-positive tumors occur most frequently in a younger group of individuals than tobacco-related malignancies. (Tobacco oral cancers occur most frequently in the fifth through the seventh decade of life.) They also

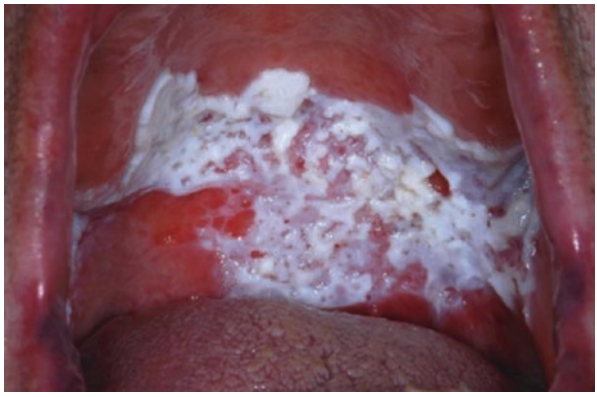

Fig. 7.4 Proliferative verrucous leukoplakia on palate

occur more in white males and in nonsmokers. The HPV-positive group is the fastest growing segment of the oral cancer population.

Treatment of PVL usually involves surgical excision and also the use of a carbon dioxide laser. Unfortunately, the lesions tend to recur and have to be excised or undergo repeated laser ablations.

Owing to the progressive nature of proliferative verrucous leukoplakia (PVL), many forms of therapy used for the management of traditional leukoplakia have been disappointing. Carbon dioxide laser, radiation, topical bleomycin solution, oral retinoids, beta-carotene, and systemic chemotherapy have all failed at achieving permanent cure. Although improvements have been noted with some of these modalities, recurrence rates after cessation of therapy are high, often within months of discontinuation of treatment.

Laser ablation reportedly has been successful in a very small group of patients followed for 6–178 months [42, 43]. Topical photodynamic therapy also may prove useful; it causes relatively low morbidity and no scarring, and multiple mucosal sites can be treated simultaneously. However, multiple treatments over the course of the disease's progression may be required. Another conflicting evidence suggests that despite the use of lasers the recurrence is as high as 83 % [43].

Inosine pranobex (Isoprinosine or Methisoprinol) is a synthetic agent capable of inhibiting viral RNA synthesis and replication and of stimulating antiviral cell-mediated reactions that have been shown to have some clinical efficacy in HPV-induced lesions. In an open-label trial of HPV-positive patients with proliferative verrucous leukoplakia treated with surgery alone versus surgery with presurgical and postsurgical treatment with Methisoprinol at 500 mg q4h for 3 days preoperatively, followed by 500 mg bid for 2 months postoperatively, 72 % and 16 % of patients in each respective treatment arm experienced relapse at 18-month postoperative follow-up; however, no longer-term follow-up or randomized controlled trial data are available [42].

7.6 Oral Submucous Fibrosis

Oral submucous fibrosis (OSF) was first reported by Schwartz in 1952 as "atrophia idiopathica mucosae oris" in five Indian women in Kenya and Joshi SG subsequently coined the term oral submucous fibrosis in 1953 [44, 45]. OSF is a disease of the oral cavity resulting from inflammation and progressive fibrosis of the submucosal tissue (connective tissue) resulting in significant rigidity and inability to open the mouth. OSF is predominantly found in Southeast Asia and caused by chewing areca nut, which is the predominant component of betel quid. The most common site involved is the buccal mucosa; however any component of the oral cavity can be involved. Arecoline, an alkaloid found in the areca nut, promotes salivation, stains saliva red, and is a stimulant.

There is a direct dose-related response of development of submucous fibrosis and frequency and duration of chewing areca nut [46]. Arecoline can promote fibroblasts to increase collagen in the submucosa by 150 % [47]. Clinically, OSF is

characterized by a burning sensation, with stiffening of the oral mucosa and oropharynx leading to significant trismus. The fibrosis and hyalinization occur in the lamina propria with resultant atrophy of the epithelium leading to predisposition to the development of squamous cell carcinoma. The annual malignant transformation rate is approximately 0.5 % [48].

Management of OSF ranges from medical management for early lesions and surgery for advanced lesions with significant trismus. The predominant goal for such patients is to improve their mouth opening. Medical management can include intralesional steroid injections combined with hyaluronidase that has proven effective [49]. Interferon-gamma also plays a role in treatment of OSF due to its immunoregulatory effect through its antifibrotic cytokine [50].

Surgical treatment described in the literature ranges from buccal fat pad reconstructions to the more advanced techniques as the utilization of radial free forearm flaps bilaterally to treat the effects of trismus. However even despite these treatments, trismus can continue to develop without continued physical therapy

7.7 Management of Oral Potentially Malignant Lesions

A firm diagnosis is required prior to initiating treatment. This often requires clinical examination, removal of potential offending agents and reexamination, incisional biopsy, and when indicated excisional biopsy.

The management of OPML is dependent on the potential risk of malignant transformation. The malignant transformation risk of OPML varies from 1 to 42 % [51]. The reported annual transformation rate ranges from 0.3 to 1 % [52]. The risk stratification is carried out at the clinical level and at histologic level. The high-risk factors are female gender, long duration of lesion, absence of risk factors, high-risk locations like the lateral border of the tongue, ventral tongue, floor of mouth, nonhomogeneous lesions, presence of *Candida Albicans* in histology, and presence of dysplasia.

Treatment of a red or white lesion involves taking into account several factors that starts with a good account of a history and clinical exam. The site and size with appearance and duration are important factors to account for during clinical examination. The right questions could identify potential irritants and risk factors. Generally a benign examination with low suspicion for malignancy is usually followed periodically. Potentially malignant disorders can be divided into high-risk and low-risk lesions. Typically, high-risk lesions should be biopsied as soon as possible to establish a diagnosis, and a lower risk lesion can be followed for 2 weeks to allow for resolution of the lesion after removal of the irritant. Persistent lesions thereafter should be biopsied (Fig. 7.5).

Once a biopsy has been completed and a diagnosis established, the treatment is again grouped into high-risk or low-risk lesions. High-risk lesions have a higher rate of malignant transformation and include moderate and severe dysplasia and carcinoma in situ, whereas the low-risk lesions include hyperplasia, hyperkeratosis, no dysplasia, or mild dysplasia. Low-risk lesions are typically followed with removal

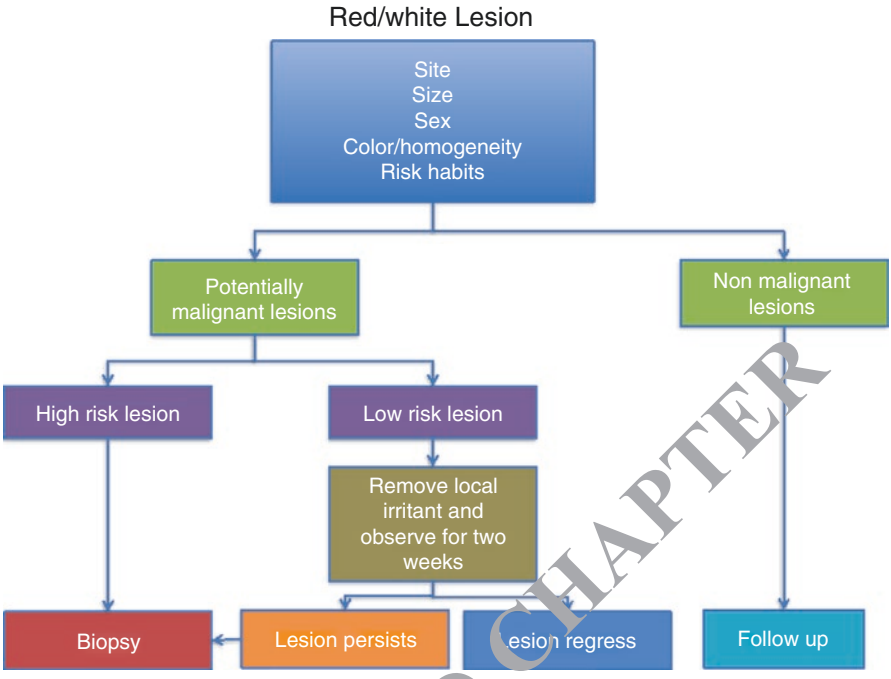

Fig. 7.5 Algorithm for diagnosis of red/white lesions

of the irritants/risk factors to allow for resolution of symptoms. High-risk lesions are usually removed and followed long term to ensure the lesions do not recur (Fig. 7.6).

7.8 Pathology of Oral Potentially Malignant Lesions

Although presence of dysplasia in biopsy is a critical factor in determining treatment plan, it has various limitations. Unlike cervical cancer, criteria to define dysplasia are not clearly defined. The histologic criteria for dysplasia depend on both tissue architecture and cytology [33]. The tissue architecture that denotes dysplasia is disorganized epithelial stratification, loss of polarity of basal cells, bulbous rete pegs, increased number of mitotic figures, mitoses in the superficial layers of epithelium, abnormal keratinization within individual cells, and presence of keratin pearls within the epithelium. The cytological features suggestive of dysplasia are variation in nuclear size and shape, variation in cellular size and shape, increased nuclear-cytoplasmic ratio, increased number and size of nucleoli, nuclear hyperchomasia, and atypical mitosis. It is to be noted that there exists no guideline on how much weightage to be given to these various factors. This may have led to high intra- and interobserver variability often seen in the pathological interpretation of oral dysplastic lesions.

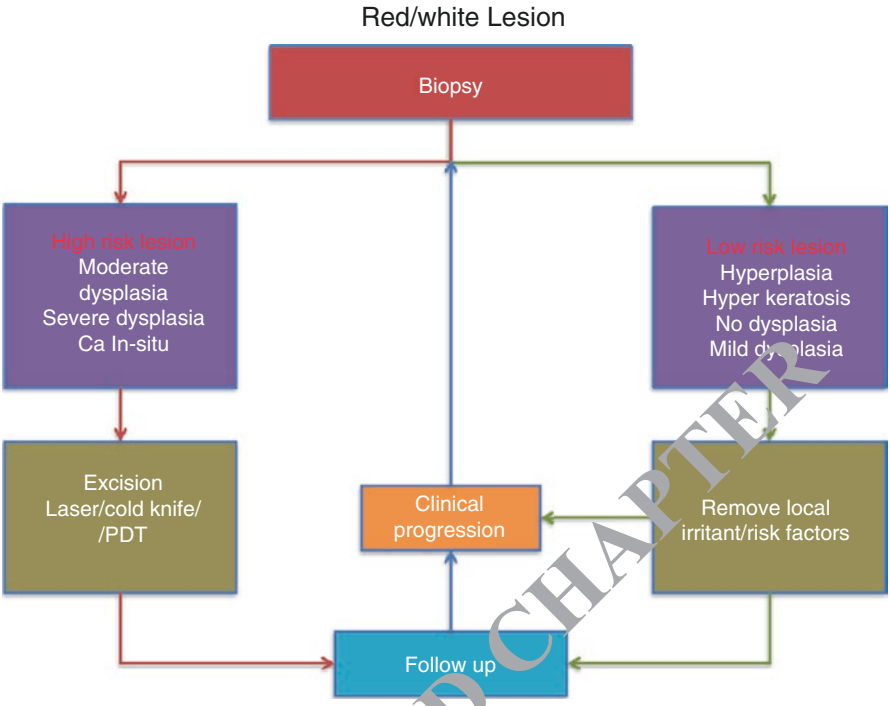

Fig. 7.6 Algorithm for management of high-risk vs low-risk red/white lesions

It is also observed that incision biopsy often underestimate grade of dysplasia or even the presence of malignancy within a lesion. In a study by Holmstrup et al. [53] involving 101 lesions in 96 patients with oral leukoplakia, all the patients after incision biopsy underwent surgical excision. Histology of the surgical specimen showed presence of invasive cancer in 7 (7 %) and 70 lesions (69 %) with dysplasia or in situ carcinoma. Comparison of biopsy and surgical specimen showed discordance of 79 % with one degree up or down the scale of epithelial dysplasia.

Although presence of dysplasia is marker of potential malignant transformation, it has been demonstrated that absence of dysplasia does not guarantee lack of malignant transformation potential and presence of dysplasia does not confer malignant transformation. Long-term malignant transformation potential was studies in cohort of 236 patients with 269 lesions [52]. Following surgical excision of 94 lesions, 11 (12 %) developed carcinoma after a mean follow-up on 7.5 years. The frequency of malignant transformation was similar in lesions with and without dysplasia and different grades of dysplasia. In the nonsurgical group of 175 lesions, 16 % regressed spontaneously and 4 % developed carcinoma during a mean follow-up period of 6.6 years. The malignant transformation rate was 14 % of dysplastic lesions and 2 % of lesions without dysplasia.

7.9 Treatment Options of OPML

The treatment options of a patient with OPML consist of (1) habit cessation, (2) surgical removal, (3) systemic medication, (4) topical medication, (5) photodynamic therapy, and (6) combination treatment.

Habit cessation Majority of oral leukoplakia is associated with tobacco, araca nut chewing, and alcohol abuse. It has been shown that habit cessation along leads to 43–58 % clinical response [54, 55]. Spontaneous regression of oral leukoplakia also has been recorded in 16 % of the lesions [52].

Surgical Treatment Although there are no prospective clinical trials to support surgical intervention of oral leukoplakia, this is the most commonly employed intervention. Surgical intervention required either as treatment or to establish accurate histologic diagnosis. Surgery has a relapse rate of 10–20 % and malignant transformation rate of 3–9 %. In Holmstrup study of 269 lesions, malignant transformation rate after surgery was 16 % after surgery and 4 % without surgery after a median follow-up of 7.5 and 6.6 years. It is quite likely that clinically and histologically more advanced lesions may have been subjected to surgical intervention [52].

Chemoprevention Chemoprevention is defined as the administration of agents to block or reverse carcinogenesis [56, 57]. The goals of chemoprevention are directed toward reversing premalignant lesions and preventing second primary cancers. Oral cancer is a unique model for developing chemoprevention agents as it has a well-defined tumor progression model from normal epithelium, mild, moderate, and severe dysplasia to carcinoma in situ and to invasive cancer [58]. Several agents have been studied including retinoids, beta-carotene, vitamin E, selenium, and Cox-2 inhibitors. The results of chemoprevention trials are summarized in Table 7.2.

Retinoids, a vitamin A derivative, are the most widely studied chemoprevention agent in oral cancer. The first landmark trial conducted by Hong et al. showed effective in treatment of leukoplakia with retinoids in 50 % of patients compared to placebo controls and they also demonstrated subsequently that retinoids can be used to prevent second primary tumors in 24 % of patients [61, 62]. Unfortunately, retinoids have a high toxicity leading to adverse effects like dermatitis, conjunctivitis, and hyperglyceridemia in a significant number of patients. Subsequently, lower doses of retinoids have been used successfully in the treatment of leukoplakia and also prevent second primary tumors.

Biochemoprevention agents have also been used for preventing and treating head and neck premalignancies and prevention of second primary tumors. Selenium has been investigated in nonrandomized clinical trials for treatment of leukoplakia [63]. Retinoid-resistant leukoplakias treated with alpha-tocopherol has produced a response rate of 46 % in a study conducted by Chiesa et al. [64]. Beta-carotene and vitamin C have been studied in the management of leukoplakias; however a recent randomized controlled multicenter trial by Nagao et al. showed no efficacy of either of the agents [65].

Table 7.2 Summary of results of systematic reviews and meta-analysis of chemoprevention trial of oral leukoplakia

Study	Type of study	Intervention	Dose	Duration	Number of patients	Clinical response – complete or partial (%)	Histologic response (%)	Recurrence	Malignant transformation (%)	Adverse effects
Shah JP (1983)	Phase II four arm	Topical vitamin A	1 mg, 3 mg, 5 mg, 10 mg	6 months	16	81.8	NR	66.6	NR	Well tolerated
Hammersley N (1985)	NR	Topical bleomycin	0.5 %	12–15 days	6	100	Improvement in dysplasia	NR	NR	Well tolerated
Hong WK (1986)	Phase II placebo controlled	13 cis-retinoic acid	1 to 2 mg/kg/day	months	44	67	54	56	NR	Cheilitis, conjunctivitis, hypertriglyceridemia
Stich HF (1988)	Phase II placebo controlled two-arm study	Systemic vitamin A	200,000 IU/week	6	53	57	50 (cytological response)	NR	NR	Well tolerated
Garewal HS (1990)	Single-arm phase II	Systemic beta-carotene	30 mg/day	6	24	1	NR	NR	8	No adverse effect
Toma S (1992)	Single-arm phase II	Systemic beta-carotene	90 mg/day	7	23	44.4	NR	5 %	NR	No adverse effect
Brenner SE (1993)	Single-arm phase II	Systemic alpha-tocopherol	400 IU/twice daily	6	43	46	21	NR	NR	Well tolerated
Tradati N (1994)	Phase II, single arm	Topical fenretinide	NR	1 month	8	100	NR	NR	NR	Well tolerated
Epstein JB (1994)	Phase III, placebo controlled	Topical bleomycin	1 %	14 days	22					

(continued)

Table 7.2 (continued)

Study	Type of study	Intervention	Dose	Duration	Number of patients	Clinical response – complete or partial (%)	Histologic response (%)	Recurrence	Malignant transformation (%)	Adverse effects
Sankaranarayanan R (1997)	Phase II placebo controlled three arm	Systemic beta-carotene, vitamin A	Vitamin A-30,000 IU/week Beta-carotene – 360 mg/week	12	131	52 (vit A) 33 (beta-carotene)	NR	75 % (vit A) 75 % (beta-carotene)	NR	No adverse effect
Liede K (1998)	Phase II placebo controlled four arm	Systemic alpha-tocopherol, beta-carotene, or both	Alpha-tocopherol 50 mg/day Beta-carotene 20 mg/day	50–84	409 smokers 24 with leukoplakia	No difference in response from placebo	NR	NR	NR	Well tolerated
Epstein JB (1998)	Phase II, single arm	Topical bleomycin	1 %	14 days	19	94	75	47.7	11	Higher risk of malignant transformation
Piattelli A (1999)	Phase II placebo controlled	Topical isotretinoin	0.1 %	4 months	10	0	NR	NR	NR	Well tolerated
Garewal HS (1999)	Phase II placebo controlled two-arm study	Systemic beta-carotene	60 mg/day	6	50	52	39	18 (beta-carotene, 17 (placebo)	8	Well tolerated
Epstein JB and Gorsky M (1999)	Case series	Topical tretinoin	0.05 % gel	23 months	26	27	30	40	NR	NR
Li N (1999)	Phase II placebo controlled	Mixed tea products	3 g/day	6 months	59	37.9	NR	NR	NR	NR

Gaeta GM (2000)	Phase II placebo controlled	Topical acitretin	20 mg/day		21	71	NR	NR	NR	Well tolerated
Singh M (2004)	Phase II placebo controlled three-arm study	Systemic lycopene	mg/day mg/day	3	58	80 (8 mg) 66.25 (4 mg)	80 (8 mg) 66.25 (4 mg)	NR	NR	No adverse effects
Mulshine JL (2004)	Phase IIb, randomized placebo controlled	0.1 % ketorolac rinse	10 ml	2 months	57	32	No significant histologic response	NR	NR	Pain, toxicity grades 1 and 2
Lippman SM (2006)	Phase II, single arm	Systemic fenretinide	200 mg/day	3	35	34.3	NR	75	NR	NR
Papadimitrakopoulou VA (2008)	Phase II placebo controlled study	Oral celecoxib	100 mg, 200 mg bid	3 months	89	31.3 %	28	NR	none	Well tolerated; common side effects were dizziness, diarrhea, and abdominal pain
Sun Z (2009)	Phase II, randomized	ZengShengPing (mixture of six herbal medicines)	4 tablets tid/day	8 to 12 months	59	67.8	NR	NR	NR	NR
Tsao AS (2009)	Phase II randomized placebo controlled, four arm	Green tea extract	500, 750, 100 mg/m^2	3 months	39	50 %	21.4	NR	38.4	Well tolerated, insomnia, headache, nausea, nervousness

(continued)

Table 7.2 (continued)

Study	Type of study	Intervention	Dose	Duration	Number of patients	Clinical response – complete or partial (%)	Histologic response (%)	Recurrence	Malignant transformation (%)	Adverse effects
Armstrong WB (2013)	Phase IIb, placebo controlled	Bowman-Birk Inhibitor	3 g/day	6 months	132	28	No significant histologic response	NR	NR	Well tolerated, palatability issues
Mallery SR (2014)	Phase II placebo controlled	10 % (w/w) freeze dried blackberry	05 g, qid	6 weeks	20	15	15	72.7	5	NR
Nagao T (2015)	Phase III randomized control trial	Beta-carotene and vitamin C	Beta-carotene 10 mg , vitamin C 500 mg/day	12 months	46	17.4	NR	NR	8.6	No significant side effects
Present study	Phase IIb randomized control trial	Curcumin	3.6 g/day	6	223	67.5	22.5	7.7	NR	Well tolerated

References: Ribeiro et al. [59] and Lodi and Franchini [60]

Curcumin is a polyphenol derived from the plant *Curcuma longa*, which is commonly referred to as turmeric. curcumin is known to have protective features against malignancy through antioxidant and anti-inflammatory properties [66–68]. Curcumin is a strong inhibitor of NF-κB (nuclear factor kappa beta), which is implicated in the oncogenesis of several malignancies including head and neck cancers. Curcumin can suppress tumor initiation, progression, and metastasis of cancers [69]. Focal areas of dysplasia are reduced in the colon by curcumin and it is considered to have high potential in the treatment of premalignant lesions in the head and neck [70–72]. Preliminary data from the Amrita Institute of Medical Sciences demonstrated in a randomized trial of 223 patients that curcumin treatment was effective in the treatment of oral potentially malignant lesions in 67.5 % of patients compared to the placebo arm 55.3 % (p: 0.03).

Researchers have targeted premalignant lesions for treatment of head and neck cancers, but to date there hasn't been a single agent that could effectively treat such lesions. Molecular and cellular targets continue to be identified, however efficacy and the safety of new agents need to be validated in experimental models before clinical trials are initiated.

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Oral Submucous Fibrosis

8

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8.1 Introduction

Oral submucous fibrosis (OSF) is a chronic, insidious disease characterised by progressive submucosal fibrosis of the oral cavity and the oropharynx. The disease sometimes extends to the pharynx and upper third of the oesophagus. As the disease progresses, the resulting loss of fibroelasticity and stiffening of the oral mucosa leads to limitation in opening of the mouth of affected individuals. The presence of fibrous bands in lips, cheeks and soft palate is a hallmark of the disease [1].

8.2 Historical Perspective

OSF was first described by Schwartz in 1952 [2] among five Indian females living in Kenya, and he coined the term atrophia idiopathica (trophica) mucosae oris. Joshi (1953) [3] coined the term submucous fibrosis based on morphological characteristics of the disease. In the same year, Lal (1953) [4] recognised the widespread

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diffuse nature of the disease affecting the whole oral mucosa. A year later, Pin [5] described a case series from Taiwan (formerly Formorsa) naming the condition idiopathic scleroderma of the mouth. Several other descriptive terms have been given by subsequent authors: idiopathic palatal fibrosis and sclerosing stomatitis. The premalignant nature of the disease was first reported by Paymaster in 1956 [6].

Oral submucous fibrosis has evolved as a clinicopathological entity over many decades, with the current clinical significance being accepted worldwide following its rediscovery by late Jens Pindborg who described the epidemiology and clinicopathologic aspects of OSF [7–12]. The topic was discussed during expert symposia in London [13], in Kuala Lumpur [14] and at the World Workshop on Oral Medicine V in 2010 [15].

8.3 Epidemiology

OSF is exclusively described among populations in India, Pakistan, Sri Lanka, Nepal, Taiwan and among the Pacific Islanders, but sporadic cases have been described from Southern China, South Vietnam, Thailand (Fig. 8.1) and among migrants from the Indian subcontinent to the UK, USA and South and East Africa. Exact prevalence figures of OSF can only be extrapolated from large house-to-house surveys. The prevalence of OSF was found to be 0.36 % in the Ernakulum district in South India [16]; 3.4 % in Durban, South Africa [17]; 3.0 % in Hunan Province, China [18]; and 17.6 % in aboriginal Taiwanese [19]. The lower figure in the Indian survey may be due to strict criteria used by them that banding was necessary to diagnose OSF. Regional variations in the incidence of OSF within in the Indian subcontinent were reported by Bhonsle [20].

In reported screening programmes, 15 cases of OSF were found among 28,295 subjects screened in a field study in Sri Lanka [21] and 23 OSF cases among 10,547 screened in a community programme in Taiwan (Su et al. 2004) [22]. Rising trends

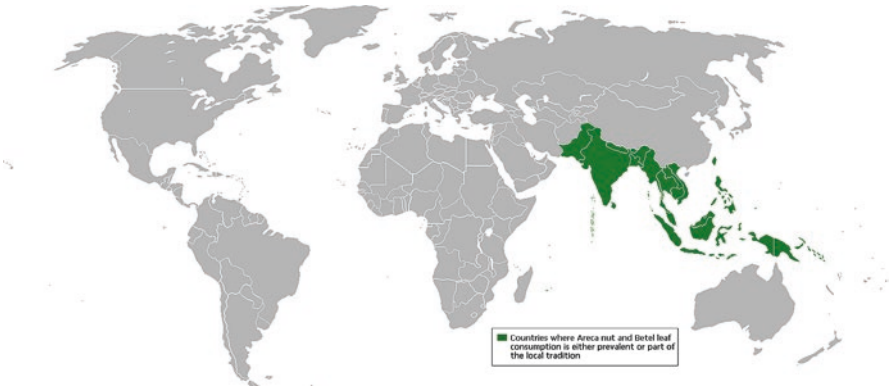

Fig. 8.1 Countries with a high prevalence of areca nut chewing habit (*marked in green*)

are reported in Gujarat, India; a prevalence of 0.16 % reported in 1967 had risen to 3.3 %, two decades later [23].

The incidence rates of OSF were reported in India by Gupta et al. [16] with a slightly higher female predilection, 19 per 100,000 in female and 8 per 100,000 in male. In a 6-year follow-up study among (aboriginal) areca/betel quid chewers in Taiwan, a higher incidence of 374.1 per 100,000 person years was reported [24].

8.4 Aetiology

Based on the epidemiological, animal and *in vitro* data from various studies assembled by the International Agency for Research on Cancer (IARC), it has been shown that areca nut is the sole aetiological factor responsible for causation of OSF. Evidence for this association is presented in several of the IARC monographs [25–27]. A plethora of other aetiological factors reported by various authors such as local irritants (chillies), nutritional deficiency and autoimmune disease are no longer considered to be causative. Areca nut (Fig. 8.2) may be consumed alone or as an ingredient of betel quid, but the role of other ingredients in betel quid (leaf, slaked lime or tobacco)[28] are not considered to be causative

Fig. 8.2 (**a**) Ripe areca fruit (**b**) Unripe areca fruit used in Taiwan (**c**) The endosperm of areca fruit, areca nut (**d**) Dried areca husks used in Souther

factors for OSF. The evidence highlighting the causative role of areca nut has been reviewed by Murti et al. [29] and Tilakaratne et al. [1, 30]. In this chapter we update the current evidence from primary studies that lead to the IARC's conclusions.

8.5 Epidemiological Studies

The evidence on the role of areca nut use in increasing the risk for development of OSF is based on case reports, case series studies, prospective cohort studies and several case–control studies conducted in India, Pakistan, Sri Lanka and Taiwan. A summary outcome of reported case–control studies conducted among several populations is presented in Table 8.1.

The first reported case control study on OSF was reported from Bhavnagar in India by Sinor et al. [31] by comparing chewing habits of mawa and bêtel quid of 60 OSF cases with 60 controls. The reported relative risks were 109.6 for all forms of areca nut chewing. Later, four other studies from India conducted in Nagpur, Kerala, Patna and Chennai [32–35], one study each from Pakistan [36] and Sri Lanka [37] and six studies from Taiwan [19, 38–42] have indicated a significant association of OSF with areca nut chewing or betel quid use. Two other studies from India described an association without showing statistical evidence [43, 44].

There are also case series reports among Indian migrants living in other countries particularly South Africa and the UK which indicate that the prevalence and frequency of areca nut use among OSF cases is much higher than in the general population [45, 46]. The percentage of subjects with an areca nut habit reported among OSF cases was close to 100 % in these studies. Case reports that describe fibrosis in non-chewers, probably had falsified habit histories [47] or included cases of oral mucosal fibrosis arising from other inflammatory disorders [48, 49].

8.6 Dose Response

A dose response confirms a causal effect of an agent under study. In the case of areca nut and betel quid, several studies have demonstrated a dose response by examining the frequency and the duration of its use. Most studies conducted so far show an increased relative risk with longer duration of use and higher daily consumption (Table 8.2). There is also clear evidence indicating that with an upsurge of manufactured products containing areca nut (pan masala and gutka) arriving in markets in India, the disease prevalence has increased significantly [23], and OSF is being diagnosed earlier (i.e. disease developing rapidly) and at younger ages [50].

Table 8.1 Epidemiological studies confirming the association of areca/betel quid use with oral submucous fibrosis in India [31–35], Pakistan [36], Sri Lanka [37] and Taiwan [19, 38–42]

Authors, study location and publication year	Characteristics of cases	Characteristics of controls	Exposure categories	Relative risk (95 % CI)	Adjustment for potential confounders
Sinor et al. Gujarat, India 1990 [31]	60 cases	60 controls	Non-chewers	1	
			Areca nut or mawa	109.6	
Maher et al. Karachi, Pakistan 1994 [36]	157 cases Attending outpatient clinic	157 controls Attending outpatient clinic for other reasons	Non-chewer	1	
			Pan	32 (6–177)	
			Pan+TOBACCO	64 (15–274)	
			Areca nut	154 (34–693)	
Hazare et al. Nagpur, India 1998 [32]	200 cases attending Dental hospital	197 age-matched controls	Non-chewer	1	
			Areca user	49.3	
Yang et al. (2001) Pingtung 1997 [38]	312 participants (119 men, 193 women)	Out of a source population of 3623 in Mutan Country (aboriginal community)	Non-chewer	1.0	Adjusted for each other, age and gender
			Areca/BQ chewer	1.8 (0.7–4.8)	
Lee et al. (2003) Kaohsiung 1994–95 [39]	125 histologically confirmed cases of OSF (93 men, 1 woman)	876 population controls (844 men, 32 women) matched on age and sex	Never chewed	1	Adjusted for education and occupation
			Former chewer	12.1 (2.8–51.9)	
			Current chewer	40.7 (16.0–103.7)	
Jacob et al. (2004) Kerala, India [33]	170 oral submucous fibrosis	47,773 subjects with no oral mucosal disorders from the same screening study	Non-chewer	1.00	
			Chewer (betel quid only)	56.2 (21.8–144.8)	
			Chewer (betel quid with tobacco)	73.0 (32.9–162.2)	
Yang et al. (2005) Mutan community, Taiwan [19]	62 subjects Detected by screening	62 controls selected from the same screening programme	Non-chewer	1.00	Non-smoker
			Chewer (betel quid only)	4.51 (1.20–16.94)	

(continued)

Table 8.1 (continued)

Authors, study location and publication year	Characteristics of cases	Characteristics of controls	Exposure categories	Relative risk (95 % CI)	Adjustment for potential confounders
Ranganathan et al. (2004) Chennai South India [34]	185 cases	185 hospital-based controls	Non-chewer	1	
			Chewer AN	3.10 (0.83 ± 11.65)	
			Chewer (pan masala)	81.50 (4.95–1341.12)	
Chung et al. (2005) Taiwan [40]	17 cases	1075 subjects examined	Non-chewer	1.00	Included smokers
			Areca quid	151.9 (19.1–999)	
Ariyawardana et al. (2006) Sri Lanka [37]	74 (61 men, 13 women) Hospital-based	74 (61 men, 13 women)	Non-chewer	1.00	Non-tobacco or alcohol consumption
			Areca nut	11.79 (0.64–217.2)	
			Betel quid	16.24 (5.8–44.8)	
Chen et al. (2006) Taiwan [41]	23 cases of submucous fibrosis (among 113 pathology archives)	23 control and 27 cases of non-premalignant disorders	Non-chewer	1.00	
			Chewer (betel quid only)	4.2 (0.17–0.54)	
Ahmad et al. (2006) Patna, India [35]	157 oral submucous fibrosis cases, hospital based	135 hospital-based controls with other diseases	Areca nut only	172.8 9(15.87–723.27)	
			Pan masala	138.21 (32.97–629.34)	
			Paan	41.5 (12.33–156.59)	
			Gutka	234.9 (67.17–900.330)	
Yen et al. (2008) China, Taiwan [42]	441 oral submucous fibrosis	8360 men Participating in a screening	Occasional use	1	Age, education, occupation, smoking and alcohol drinking
			+20 pieces/day	6.89 (4.96–9.58)	

Adapted from IARC Monographs 2004, 2012, Modified from Tilakaratne et al. [1]

Table 8.2 Dose–response relationship of areca habits and OSF

	Quids/day	Odds ratio (95 % CI)	Duration of chewing (years)	Odds ratio (95 % CI)
Maher et al. (1994) [36]	0	1	0	1
	1–5	84 (20–360)	1–5	72 (17–316)
	6–10	246 (47–1278)	6–10	137 (29–640)
	10+	100 (19–522)	10+	109 (25–479)
Yang et al. (2001) [19]	1–10	1.0	1–10	1
	11–20	1.2 (0.7–2.04)	11–20	1.8 (0.7–4.8)
	>21	1.3 (0.7–2.2)	21–30	2.4 (1.0–5.0)
			>31	2.4 (1.1–5.0)
Lee et al. (2003) [39]	1–10	31.4 (11.9–82.5)	1–10	30.9 (11.3–84.7)
	11,020	37.4 (12.6–110.4)	11–20	41.9 (14.1–124.9)
	>21	53.5 (16.4–174.8)	>21	39.3 (11.7–131.7)
Yang et al. (2005) [38]	1–9	3.66 (0.71–18.91)		
	10–29	4.55 (1.16–17.84)		
	30+	10.34 (2.30–44.73)		
Yen et al. (2008) [42]	Occ	1		
	1–10	1.26 (0.91–1.74)		
	11–20	3.88 (2.75–5.60)		
	20+	6.98 (4.96–9.58)		

8.7 Genetic Predisposition

There are over 600 million areca nut chewers reported worldwide [27]. However, only 1–2 % of the population may develop the disease. This suggests a possible genetic predisposition in the affected people. Rapid development of OSF in young adults or even children reported in clinical case reports [51] further adds weight to this hypothesis. Genetic polymorphisms discovered in affected individuals that may predispose them to the disease are discussed in a later section in this chapter.

8.8 Experimental Animal Studies

In vivo experimental data on the ability of the areca nut extract to produce OSF is meagre. However, Huang, Ling and Wu [52] claim to have produced a rat model of OSF in Hunan Medical University, China, and earlier in vivo experiments of Khrime et al. [53] showed histopathological findings akin to OSF induced by *pan masala* on the rat mucosa. The characterizations of these models were not complete and the experimental evidence was neither convincing nor

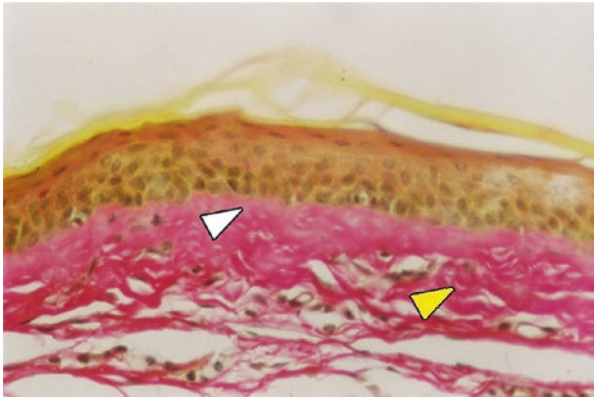

Fig. 8.3 Histopathological features of OSF, illustrating thin atrophic epithelium and fibrosis of underlying connective tissue. *Arrows* show collagen deposition

reproducible. The relevance of a particular animal model to a human disease rests on its ability to parallel the biological changes that characterise the disease in humans.

Perera et al. [54] described an OSF animal model in female albino mice of BALB/c strain. They applied an aqueous areca nut extract prepared from fresh, mature endosperms of *Areca catechu* by dissolving nuts in 0.9 % normal saline (50 mM NaCl) on the buccal mucosae of mice ($n = 40$) for 600 days. Their study showed fibrosis of treated buccal mucosa as a continuous process occurring in the subepithelial buccal mucosal tissues of treated mice (Fig. 8.3). The amorphous areas confirmed by van Gieson and Masson's trichrome stains were an indication of early hyalinization and reflected the presence of young collagen or altered ground substance or both. These findings confirming the excessive deposition of collagen in the treated animals did bear a close similarity to human OSF.

In this *in vivo* mouse model, the effects of areca nut extract on epithelial thickness leading to atrophy, connective tissue fibrosis, progressive reduction of fibroblasts and inflammatory changes were closely similar to that found in human OSF [54]. The experimental data presented by Perera et al. further supports areca nut contributing to the causation of OSF.

8.9 *In Vitro* Studies

Several investigators have studied the effects of constituents of areca nut, such as arecoline and arecaidine, on oral fibroblasts *in vitro* in order to provide corroboratory evidence of cause and effect. The addition of arecoline and arecaidine has shown stimulatory effects on fibroblasts in culture [55–57]. In a later study, fibroblasts when subjected to different concentrations of aqueous concentrations of raw or boiled areca nut showed morphological alterations [58]. In other *in vitro* studies, fibroblasts from OSF specimens showed more than a 1.5-fold increase in production

of collagen compared with fibroblasts from age- and sex-matched and passage-matched normal controls [59].

8.10 Summary on Aetiology

A comprehensive evaluation of above data led the IARC [25, 27] to confirm the aetiological role of areca nut as the causative agent of OSF. In our wide experience from field and clinical studies, we have not encountered any single case of OSF in a non-areca nut chewer. Few case reports that describe OSF in white Caucasians [48, 49] appear to be a misclassification of the disorder due to finding of sclerotic fibrous bands rarely encountered in other chronic inflammatory disorders (e.g. ulcerative lichen planus or chronic oGVHD).

8.11 Aetiopathogenesis

Although the disease was described in 1950s, its pathogenesis has not been clear until recently. Three published reviews [30, 60, 61] had undertaken to critically examine the scientific data available on the pathogenesis of OSF published up to 2015. Several mechanisms and biological pathways have been proposed for the pathogenesis of the disorder, all based on the constituents of areca nut and genetic susceptibility to the disease. The flow chart shown below illustrates the possible biochemical and molecular events known in the pathogenesis of OSF (Fig. 8.4 – modified from WWOM V) [15].

8.12 Mechanisms of Pathogenesis of Oral Submucous Fibrosis

8.12.1 Constituents of Areca Nut and Their Primary Effects

Areca nut contains active components including alkaloids (arecoline, arecaidine, guvacine, guvacoline and arecolinidine), polyphenols (catechin, flavanoids, flavan-3:4-diols, leucocyanidins, hexahydroxyflavans and tannins) and trace elements (sodium, magnesium, chlorine, calcium, vanadium, manganese, copper and bromine). Arecoline was identified as the principal causative factor for OSF by Caniff's group [55, 56] and appears to be involved in the pathogenesis of OSF by causing fibroblastic proliferation and increased collagen formation. It appears that the main pathological change in OSF is the increased accumulation of type 1 collagen within the subepithelial tissues. Polyphenols of areca nut such as flavanoids, catechin and tannins cause collagen fibres to cross-link and thereby make them less susceptible to collagenase degradation. The resulting decrease in collagen breakdown in turn leads to increased fibrosis which is the mainstay of the pathogenesis of OSF [30]. In the past decade, various mechanisms leading to submucosal fibrosis have been demonstrated and these are briefly presented.

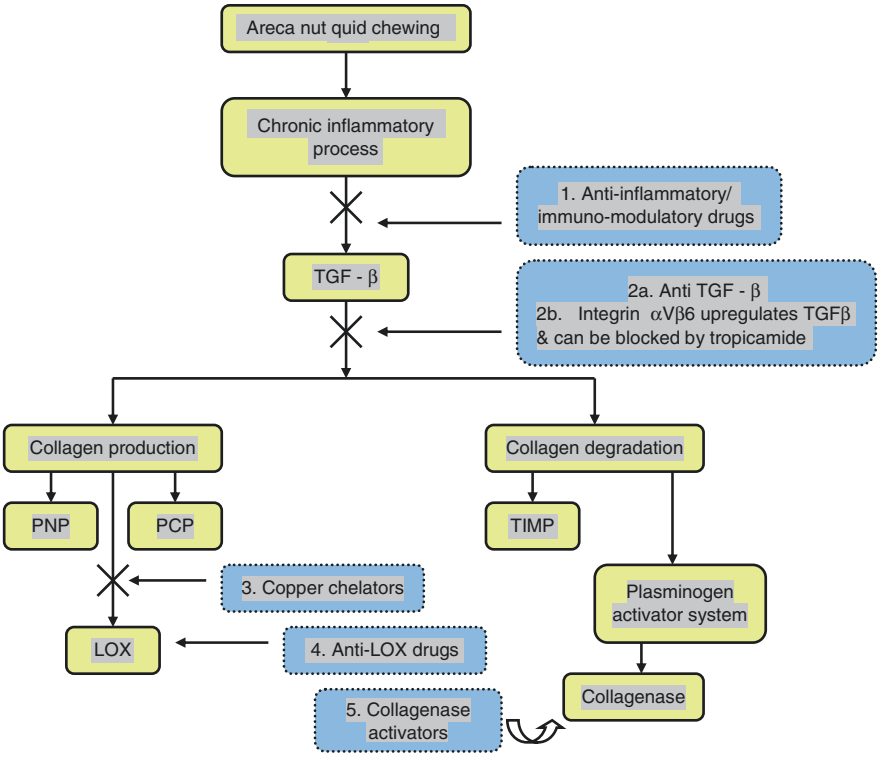

Fig. 8.4 Pathogenesis of oral submucous fibrosis (OSF) – A schematic illustration of the collagen production pathway and potential elements of molecular interventions (Modified from Rajalalitha and Vali [60]). *PCP and PNP* – the enzymes known as the procollagen C and N proteinases (PCP and PNP) are involved in the processing of fibrillar procollagen precursors to mature collagens. *TIMP* – the matrix metalloproteinases are inhibited by specific endogenous tissue inhibitors of metalloproteinases (TIMPs), which comprise a family of four protease inhibitors

8.12.2 Fibrogenic Factors

Several fibrogenic cytokines such as transforming growth factor-β1 (TGF-β), basic fibroblastic growth factor (bFGF) and connective tissue growth factor (CTGF) are associated with fibrosis of different organs. Among these TGF-β is known to be a potent stimulator of extracellular matrix through inducing transdifferentiation of fibroblasts into myofibroblasts [62] (Fig. 8.5). TGF-β has been shown to be expressed in OSF tissues [63–65] (Haque et al. 1998), and the key role of this cytokine in the progression of OSF has been proposed by other authors (Khan et al. 2012) [66]. Several studies have shown that αγβ6-dependent TGF-β1 activation promotes pathogenic organ fibrosis. Moutassim et al. (2011) [67] have demonstrated upregulation of αγβ6 in OSF tissue samples, and their study indicates this as the likely mechanism involved in TGF-β1 activation in OSF by arecoline, leading to

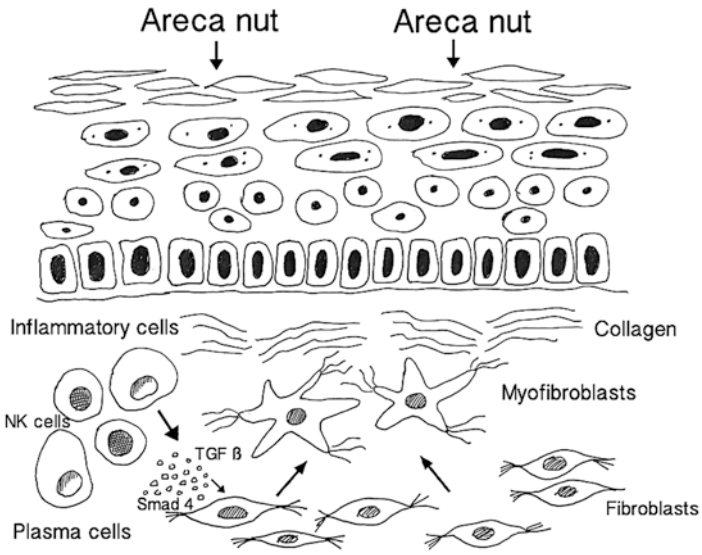

Fig. 8.5 Schematic diagram of fibroblast activation (courtesy of Dr Helen McParland)

fibrosis. Several other growth factors may also be upregulated in OSF such as basic fibroblast growth factor [68] and insulin-like growth factor-1 [69].

Involvement of connective tissue growth factor (CTGF) in fibrosis in many human tissues is well established [70]. Expression of CTGF (ccn2) was reported in fibroblasts in scleroderma patients [71], and a further study has shown that arecoline stimulated CTGF production in buccal mucosal fibroblasts through reactive oxygen species (ROS) [72]. Both CTGF can induce collagen production via TGF-β dependent and independent pathways. However, recent work by Kahn et al. suggests a major causative role for TGF-β that is induced by areca nut in OSF progression [73] (Fig. 8.5).

Experimental studies have shown that mechanical trauma may also induce secretion of TGF-β (Manokawinchoke et al. 2015) [74]. Thus continuous mastication (of areca nut) may have a similar effect and contribute to TGF-β deposition in buccal tissues.

8.12.3 Matrix Metalloproteinases and Tissue Inhibitors of Matrix Metalloproteinases (MMPs and TIMPs)

Accurate and balanced collagen metabolism is essential to maintain the normal integrity of connective tissue. Equilibrium between two enzyme groups, MMPs and TIMPs, is mandatory to achieve the above. In OSF, the equilibrium between MMPs and TIMP is disturbed in such a manner that it ultimately results in increased deposition of extracellular matrix ECM. Immunohistochemical studies have shown that

MMP-1 expression is attenuated in OSF compared to normal oral mucosa [64, 75]. Since MMP-1 is the main human enzyme that degrades fibrillar collagen, this suggests that collagen degradation caused by MMP-1 is reduced in OSF [75]. In addition, stronger intensity of TIMP-1 in fibroblasts of OSF compared to normal oral mucosa suggested improper regulation of proteolytic equilibrium as one of the main factors responsible for the excessive fibrosis in OSF [64]. The fibroblasts in OSF have a reduced replicative life span as they accumulate senescent cells during the progression of the disease [76]. This is due to the increased amount of ROS and DNA double-strand breaks (DDBs) produced intrinsically by damaged mitochondria. TIMP-1 and TIMP-2 are increased in fibroblast cultures of OSF relative to normal and non-diseased paan user controls [77].

8.12.4 Copper and Related Structural Changes of Collagen

The role of copper in the pathogenesis of OSF was raised by the King's College Group as a result of their novel finding of high copper content in areca nut [78]. The copper-dependant enzyme lysyl oxidase is critical for collagen cross-linking and organisation of ECM [79], and this enzyme was found to be upregulated in OSF. Salivary copper is found to be higher in areca nut chewers [80]. Salivary copper levels appear to vary from mild OSF to severe cases [81]. These findings indicate that soluble copper found in areca nut is released into the oral environment and its oral absorption may contribute to fibrosis of buccal mucosa suggesting a possible local effect of copper in OSF patients [82]. Serum copper levels in OSF patients are also raised suggesting a systemic effect, and levels correlate with the advancement of the clinical stage [83, 84]. However, the effects of copper appear to be local in the context of OSF as there is no evidence to suggest that these patients develop systemic fibrosis. Spraying of areca crops with copper sulphide used as a fungicide to preserve the fruit has been attributed as a likely source of high copper in the areca growing belt.

8.12.5 Changes in the Extracellular Matrix

Histopathological evidence shows ECM remodelling with the progression of the disease. It has been reported that in early stage of OSF, tenascin, perlecan, fibronectin and collagen type III are overexpressed in the lamina propria and submucosa [85]. Extensive and irregular deposits of elastin were found around muscle fibres in the intermediate stage, together with the above molecules. In the advanced stage, collagen type I appears to dominate the ECM. The gene expression levels of these molecules were varied with the progression of fibrosis. This pattern of ECM remodelling steps in OSF is similar to normal granulation tissue formation and maturation process. Difficulty in opening the mouth may be related to the loss of various ECM molecules such as elastin and replacement of muscle by collagen type I [86].

Table 8.3 Genetic polymorphisms predisposing to OSF

Genetic polymorphism	Role in pathogenesis of OSF	References
Cytochrome P450	A genetic biomarker for susceptibility to OSF	[87, 88, 95]
Cytochrome P450 3A, P4501A1, CYP2E1	Helpful in identifying high-risk individuals	
CYP1A1(m1) and (m2)		
Genotypes	Acts as protective factors (in the absence of GSTM1 and/or GSTT1 genes), alters risk towards the disease	[89]
Lysyl oxidase		
LOX Arg158Gin	Associated more in elderly OSF patients	[90]
TGFβ-1 (single nucleotide polymorphism in 5¢UTR C-T)	Associated with pro-angiogenic pathway	[91]
MMP-3 (single nucleotide polymorphism in 1171 5A->6A)	Increased risk for developing OSF	[92, 93]
N-acetyltransferase	Increase the risk of OSF in men	[94]

8.12.6 Genetic Polymorphism Predisposing to OSF

Polymorphisms of various genes have contributed to the pathogenesis of disorders in different ways. Polymorphism of the cytochrome P450 3A gene family is considered as a major determinant of interindividual variability in chemical pharmacokinetics. Cytochrome P450 had been identified as a genetic biomarker for susceptibility to develop OSF. This may be helpful in identifying high-risk individuals according to the genetic polymorphisms in some exclusive regions of the cytochrome P450 3A, P4501A1 and CYP2E1 genes [87, 88]. The evidence available to support other possible genetic predispositions [89–94] to the disease is summarised in Table 8.3.

8.12.7 Clinical Features

A workshop held in Kuala Lumpur, Malaysia [14], recommended the following clinical criteria for the diagnosis of OSF:

- Presence of palpable fibrous bands
- Leathery mucosal texture
- Blanching of mucosa
- Loss of tongue papillae
- Burning sensation to spicy food
- Rigidity of the tongue

Blanching of the mucosa is an early feature, and some authors refer to this as depigmentation of mucosa [96]. Fibrous banding involving the buccal mucosae (Fig. 8.6), lip or palate (Fig. 8.7) is noted in established stages of OSF. At later stages, this may manifest with a marble-like appearance. Occurrence of small

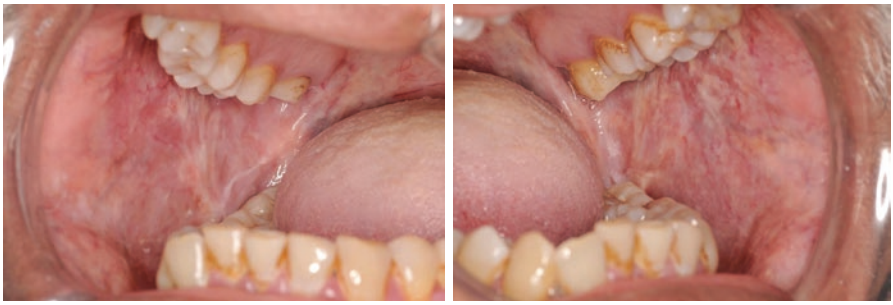

Fig. 8.6 Fibrous bands on buccal mucosa in OSF

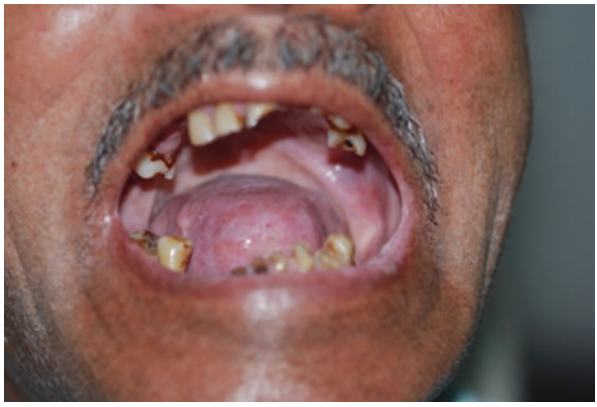

Fig. 8.7 Palatal fibrosis in OSF

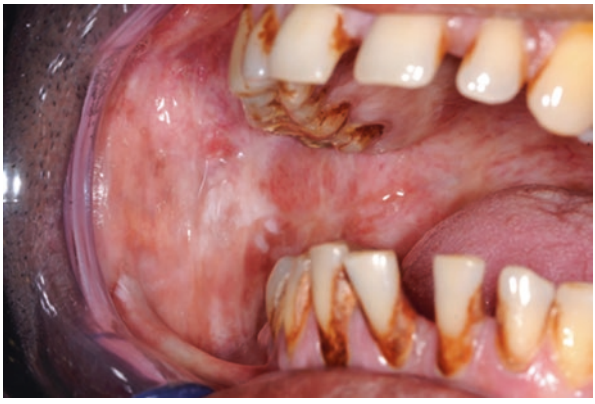

Fig. 8.8 A patch of leukoplakia in a case of OSF

reddish blue spots in a quarter of the patients was highlighted by Bhonsle et al. (1981) [97]. Presence of vesicles has also been reported as an early feature. Due to vertical bands, progressive limitation of mouth opening is a hallmark feature of this disease. Other potentially malignant disorders such as leukoplakia also caused by betel quid may be found to coexist (Fig. 8.8).

Table 8.4 OSF disease grading system proposed by Warnakulasuriya and adapted at WWOM V

Grade 1 – burning, depapillation, blanching or leathery mucosa (disease triad for OSF); mouth opening, >35 mm
Grade 2 – moderate limitation of opening 20–35 mm (+ disease triad and fibrous bands)
Grade 3 – severe OSF, limitation of opening <20 mm
Grade 4A – OSF + other potentially malignant disorder
Grade 4B – OSF with oral epithelial dysplasia
Grade 5 – OSF + SCC

8.12.8 Staging

Several classification schemes have been proposed for staging OSF. These may be based solely on clinical criteria or histopathological features; Warnakulasuriya [98] first reported to use the inter-incisal opening as a semiquantitative clinical measure to assess the worsening of OSF. An opening limited to 35 mm or less was considered as moderate or advanced disease. Maher et al. [99] tested the inter-incisal opening as a measure of severity of OSF and confirmed it correlated well with the extent of the disease found within the oral cavity. Pindborg and Sirsat (1966) proposed a classification based on histopathology [8]. Clinical classifications have been proposed by Pindborg (1989) [12], Lai [100] based on mouth opening to four groups and Ranganathan having first examined normal subjects (2001) and classifying OSF subjects (2006) [101, 102]. Pindborg's 1989 classification did not use mouth opening but staged the disease by mucosal alterations (vesicles, ulceration, pigmentation) and fibrosis. Later authors have used mouth opening as an important factor to grade the severity of the disease. The latest staging proposed at World Workshop on Oral Medicine V [15] is given in Table 8.4. This allows the clinician to monitor the disease during follow-up or to assess the efficacy of an intervention, whether the disease is stable, improving or progressive.

8.12.9 Histopathology

In a majority of OSF specimens, the surface epithelium shows thinning (atrophy) and flattening, while few may show epithelial hyperplasia due to chronic mastication of areca nut. Uniform hyalinization of the juxtaepithelial layer is a pathognomonic feature (Hamner 1974) [103]. Varying degrees of inflammation in the lamina propria with a sprinkling of lymphocytes is found. The characteristic feature is the presence of collagen in the upper part of submucosa [104]. In the early stages, plump fibroblasts may be found. The collagen bundles may extend up to striated muscle and sometimes embed within muscle fibres. The disease can be staged by the state of fibrosis seen in histological sections. However, no correlation has been noted between the clinical stage and the stage of fibrosis, as the biopsy may not be representative [105, 106]. In the early stage, the fibrosis is confined to the upper portion of submucosa. In the intermediate stage, there is subepithelial hyalinization

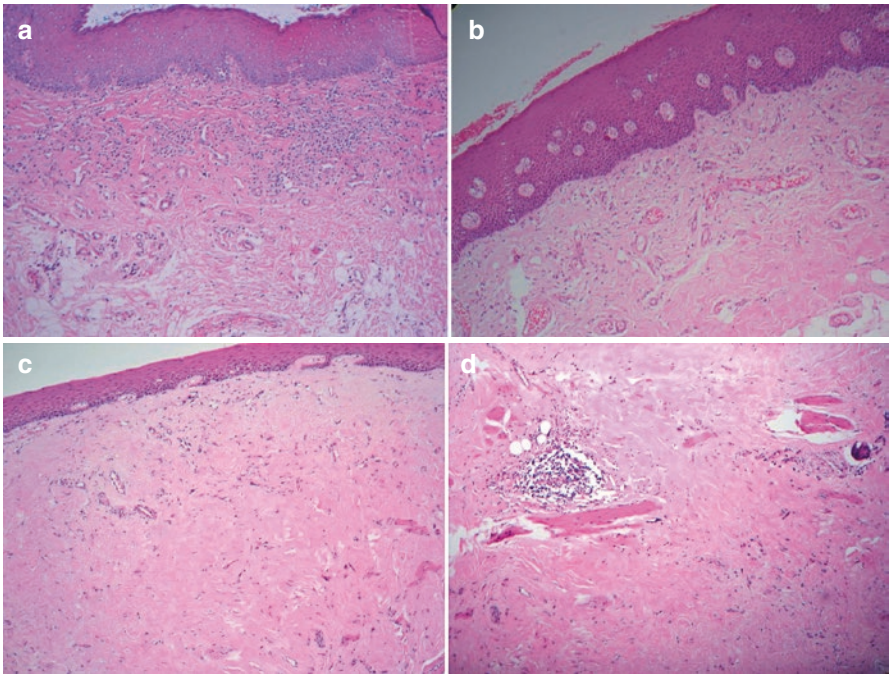

Fig. 8.9 Histopathological features of OSF. (**a**) Early stage: mild atrophy of the epithelium and fibrosis of the upper corium. Light scattered lymphocytic infiltrate within collagen fibres. (**b**) Intermediate stage: fibrosis advances into deeper corium. (**c**) Advanced stage: marked epithelial atrophy and dense fibrosis and hyalinization of the corium and replacement of muscle by fibrous tissue. (**d**) Complete replacement of muscle by fibrous tissue at the advanced stage

with fibrosis extending to deeper tissues. The advanced stage is demarcated with extensive full-thickness fibrosis of the submucosal tissue up to muscle layers together with hyaline changes [86]. Figure. 8.8 illustrates features of fibrosis as noted in the proposed staging by these authors.

In electron microscopic studies, excessive increase of collagen, especially type 1 (van Wyk et al. 1990) [107], and some necrosis of muscle have been reported [108].

Pindborg et al. (1970) [9] first reported the presence of epithelial dysplasia in a quarter of his OSF cases. In a recent study that analysed 42 OSF cases from Sri Lanka, 19 (45.2 %) showed epithelial dysplasia [64]. Figure 8.9 illustrates features of epithelial dysplasia in OSF. No significant association of presence or absence of dysplasia with the stage of fibrosis has been reported, suggesting the two processes are independent of each other. Although it has been proposed that the severity of epithelial dysplasia is proportional to the risk of subsequent cancer development [109], this has yet to be substantiated. Squamous cell carcinoma arsing from

8.12.10 Molecular Studies

Although the exact mechanisms are not clear, various chromosomal, genetic and molecular alterations are associated with the pathogenesis of OSF. An understanding of molecular events in OSF is emerging. One of the earliest attempts to characterise molecular aberrations in OSF related to detailed examination of mutations of P53 gene and its protein expression [110]. Positive p53 immunostaining was observed in 75 % of OSF cases and by PCR-SSCP novel mutations in p53 were reported in exons 2–9. In this study, 16 different mutations in p53 were found in 21 OSF samples from Karachi, Pakistan. Other key molecular features of OSF have recently been described. MMP-1 expression is reportedly attenuated in OSF while TGF-β1 expression is upregulated [64]. MMP-1 is the main human enzyme that degrades fibrillar collagen. As expected, MMP-1 levels in OSF connective tissue were attenuated compared to normal oral mucosa [75]. This shows that collagen degradation caused by MMP-1 is downregulated in the OSF, causing accumulation of ECM in the connective tissue. TGF-β is a known potent mediator which stimulates collagen and other ECM production [86]. Significantly increased TGF-β1 expression has been demonstrated in the lamina propria of OSF compared to NOM. A study using oligonucleotide microarray analysis has shown an upregulation of 716 genes and downregulation of 149 genes in OSF [111]. These genes are involved in the immune response, the inflammatory response and epithelial–mesenchymal transition (EMT) induced by TGF-β signalling pathway, namely, SFRP4, THBS1, MMP2 and ZO-1. In another study, differentially expressed genes in OSF were analysed using bioinformatic tools, and the genes were located on chromosomes 1, 2, 5, 6, 7, 11 and 12. Gene ontology (GO) classification identified these genes to be related to cellular component subgroups associated with extracellular matrix, cytoskeleton and cell membrane and also biological process subgroups associated with protein binding, signal transducer activity and immune and defence responses [112] (Figs. 8.10 and 8.11).

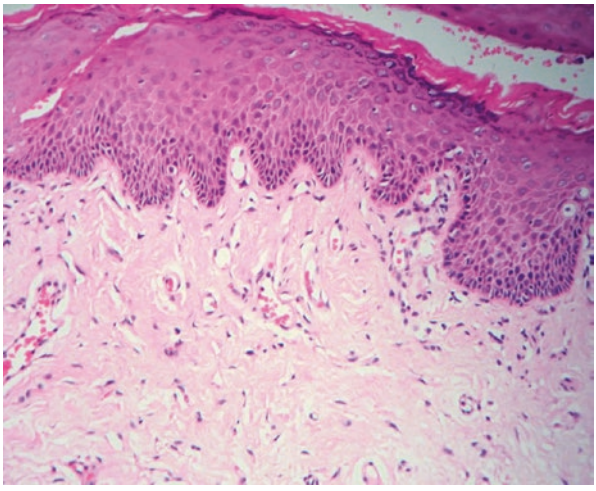

Fig. 8.10 Mild epithelial dysplasia in the background of OSF with new blood vessel formation

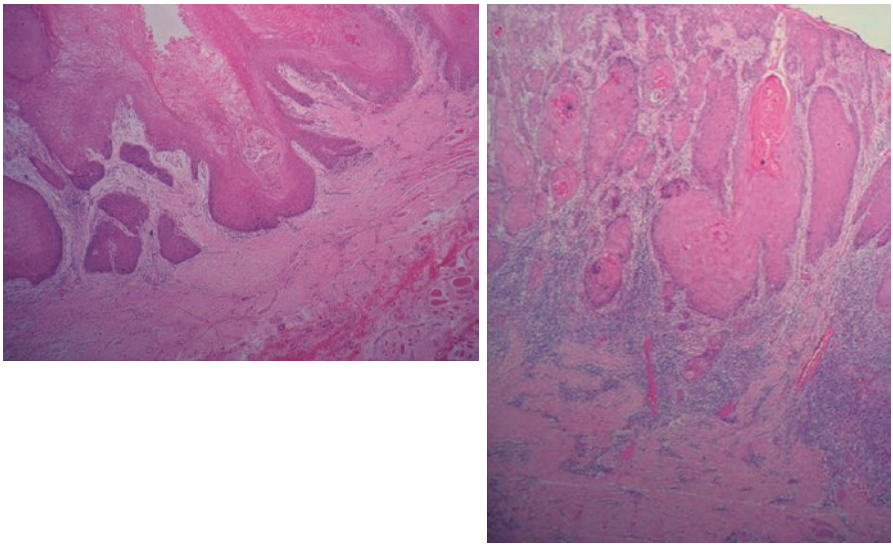

Fig. 8.11 Squamous cell carcinoma in the background of OSF

8.12.11 Malignant Transformation

The observations by Paymaster and Jens Pindborg over 50 years ago claimed the premalignant nature of OSF and provided evidence for its propensity for malignant transformation. Paymaster [6] described the development of slow-growing squamous cell carcinomas in one-third of OSF cases seen at Tata Memorial Hospital in Bombay, India. Pindborg on the other hand was observant of the coexistence of OSF in 40 % of 100 consecutive OSCC cases he reported from south India (Pindborg et al. 1966) [8].

Cancers arising in OSF are noted to be large exophytic lesions which are clinically typical OSCC without showing much histological evidence of invasion. One study reported that most of these patients are younger males showing good prognostic factors: better grades of tumour differentiation, lower rates of nodal metastases and limited extracapsular spread compared to older patients [113]. A retrospective study in China has reported contradictory data in which they state that OSCC originated from OSF is clinically more invasive and also exhibits higher rates of metastasis and recurrence rate than OSCC not originated from OSF [114].

8.12.12 Molecular Events During OSF-Carcinoma Sequence

Epithelial–mesenchymal transition (EMT) is a key mechanism in carcinogenesis. EMT has gained significant attention due to its implication in cancer and fibrosis. TGF-beta may play significant effect on EMT. Cell injury caused by areca nut extract (ANE) produces reactive oxygen species (ROS) which in turn triggers both MAPK and NF-κB pathways involved in EMT of OSF [115]. A study from our

group showed that arecoline upregulates αvβ6 expression in oral keratinocytes which in turn promotes keratinocyte migration and induces invasion [67]. It has been reported that over 80 % of OSCCs arising on a background of OSF had moderate to high αvβ6 expression [67]. We also found a statistically significant correlation with the degree of epithelial dysplasia and expression level of the gene HIF-1α that led us to conclude that hypoxia together with overexpression of HIF-1α play a role in malignant transformation of OSF [116].

Matrix metalloproteinase-2 (MMP-2) can degrade extracellular matrix and basement membrane and play an important role in the development and progression of multiple carcinomas. Subjects carrying CC genotype – a polymorphism in the MMP-2 – had nearly twofold increased risk for developing OSCC when comparing with CT or TT genotype [117].

Genomic instability in the form of LOH has been reported in OSF. This acquisition of LOH may subsequently alter gene function and expression. Several hot spots affecting LOH loci (in 47–53 % of OSF samples) have been identified, and a key finding is LOH in a large region of the chromosome 13-13q14 to 13q33. Considering the well-known fact that chromosome 13q is highly susceptible to genomic instability in HNSCC, we hypothesised that genes within the 13q14–q33 LOH region found in the OSF may play an essential role in the initiation of oral carcinogenesis in these patients. Other LOH loci revealed in this study with previously identified susceptibility regions in HNSCC include 3p24-p22, 6q26-q27, 9q22.3, 12p11.2 and 20p12-11 [118].

8.13 Management

Numerous medical interventions have been tested, but none so far have predictably shown any clinically meaningful benefit in improving mouth opening and other secondary end points. These clinical interventions were discussed at the World Workshop in Oral Medicine V and reported by Kerr et al. [15]. A wide range of medical interventions have been studied and include nutritional supplements, antioxidants, anti-inflammatory and immunomodulatory agents, biogenic stimulators, cytokines, enzymes and fibrinolytic agents and vasodilators.

Nutritional supplements (i.e. vitamin A, vitamin B, multivitamins, iron, zinc) and antioxidants (i.e. lycopene, beta-carotene, tea pigments, aloe vera and curcumin) are thought to correct deficiency states, promote tissue health and reduce the propensity for adverse effects secondary to chronic areca nut use. Anti-inflammatory and immunomodulatory agents (i.e. topical and intralesional corticosteroids, interferon gamma (IFNγ), levamisole) are thought to reduce the pro-fibrotic inflammatory pathways. Intralesional placental extracts have been hypothesised to act as biogenic stimulators, promoting regeneration of healthy tissues. Enzymes and fibrinolytic agents (i.e. hyaluronidase, collagenase and chymotrypsin) have been tested to degrade fibrotic tissues. Finally, vasodilators (i.e. pentoxifylline, nylidrin hydrochloride and buflomedil hydrochloride) have been tested to boost blood flow to the tissues. Many of these interventions have been tested in uncontrolled open label studies, and the randomised controlled trials on OSF that have been conducted so

far, have significant limitations. Most non-specific antifibrotic agents used as therapeutic regimes have been ineffective in halting or reversing fibrosis.

A few studies, with variable results, have explored the use of physiotherapy, either alone or as an adjunct to medical and surgical therapies to increase opening through tissue remodelling.

For advanced cases various surgical treatments with numerous different types of flap reconstruction have been reported. Immediate outcome are generally excellent, although there is often relapse in mouth opening. Physiotherapy undertaken immediate post-surgery could sustain the noted improvement. Finally, there is very little research exploring the impact of habit cessation on these interventions.

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9 Advances in Early Detection and Diagnostic Adjuncts in Oral Cavity Cancer

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9.1 Introduction

Oral and pharyngeal cancers combined rank within the top ten most common malignancies in the world for men, with an estimated global incidence of oral cancer alone at approximately 275,000 [1]. Over 90 % of oral cancers affecting the lips, gingiva, tongue, buccal mucosa, floor of mouth and hard palate arise from the squamous epithelium and are thus termed oral squamous cell carcinomas (OSCCs) [2, 3]. Conversely, neoplasms originating from the epithelial lining of oropharynx are called oropharyngeal squamous cell carcinomas (OPSCCs) [1].

Oral cancer ranks as the sixth most common malignancy worldwide with an estimated 263,900 new cases and 128,000 deaths in 2008 alone [4]. Patients with OSCC are typically males over 40 years of age with a history of regular exposure to

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aetiological risk factors such as tobacco products, alcohol, betel quid or micronutrient deficiency [2, 5]; however younger patients with lower cumulative tobacco or alcohol exposure are increasingly presenting with OSCC or OPSCC [2]. These early-onset OSCCs or OPSCCs are often located at the base of the tongue, tonsils and oropharynx, and are associated with the human papillomavirus infection [1, 2, 6, 7]. Alcohol and tobacco have a synergistic effect with heavy drinkers and smokers having 38 times the risk of developing oral cancer compared to those who refrain from both. Worryingly, current trends show an increase in the incidence of oral cancer among several populations regardless of an increase in knowledge about aetiological and risk factors for OSCC [1, 8, 9]. Despite technological advances in cancer therapies, the 5 year survival rate for oral cancer remains at 50 % for most populations and has not changed significantly for the past three decades [1].

The presence of synchronous or metachronous OSCCs due to the field cancerisation effect further reduces prognosis [10–12]. Early detection of OSCCs at the dysplasia or carcinoma in situ (CIS) stages improves morbidity and mortality as there is a very low risk of metastasis [13–15]. Consequently, painful, invasive and disfiguring treatment that often results in loss of function and reduced quality of life can be avoided [15, 16]. To illustrate, late stage detection is typically associated with approximately three times greater treatment costs [17] ($133,000 vs $50,000 for late vs early stage cancers, respectively) and recurrence rates (57 vs 20.3 % for late vs early stage cancers, respectively) [18]. However, it can be difficult to detect OSCC in the early stages as they are not only relatively asymptomatic but can also have very subtle changes in the epithelium that make them difficult to visualise with standard visualisation techniques using white light (WL) inspection [15, 19]. These mucosal changes may appear as patches of white, red or speckled red-white, and are called leukoplakia, erythroplakia or erythro-leukoplakia (speckled erythroplakia), respectively, when there is no clinical or histopathological diagnosis [2].

The poor prognosis for oral malignancies can largely be attributed to the late stage of diagnosis of these cancers. Patients with a delayed diagnosis of oral or oropharyngeal carcinoma are 30 % more likely to present with an advanced stage tumour compared to those without a delayed diagnosis [20–22]. The TNM classification system is widely used to delineate the extent and spread of a cancerous lesion and there is a significant decrease in prognosis with a more advanced TNM stage at initial presentation. The 5 year survival rates for stage I and II tumours are 85 and 66 % respectively, while this decreases for stage III and IV tumours to 41 and 9 % respectively [23]. Almost half of all oral cancers are diagnosed at stage III or stage IV despite the fact that these lesions present in parts of the anatomy that are easily visualised by medical or dental practitioners [20]. As such, an emphasis should be placed on the earlier diagnosis of these cancers.

Squamous cell carcinoma is often preceded by lesions such as leukoplakia or erythroplakia which have the potential to progress to malignancy. Lesions which have the potential to progress to malignancy are referred to as oral potentially malignant disorders (OPMDs). The key to improved patient prognosis is believed to be through early detection of these lesions [5, 24]. Detecting dysplastic changes at an early stage allows for active intervention before they progress to malignancy.

Other conditions such as oral lichen planus (OLP) and submucous fibrosis are also considered to be potentially malignant disorders [25–28]. There is also evidence that chronic hyperplastic candidosis may also induce dysplastic changes in oral epithelium [29, 30]. Current practice for the detection of malignant or potentially malignant lesions involves a conventional oral examination (COE) with visual and tactile examination by the dental practitioner, with leukoplakia or erythroplakic lesions considered suspicious for oral epithelial dysplasia (OED) or OSCC [31–34]. Induration and fixation in particular are tactile signs which may suggest oral malignancy. To confirm clinical findings, patients are usually referred to a specialist centre for biopsy of lesions for a definitive diagnosis and management. A biopsy is considered the gold standard for the diagnosis of OED and OSCC as it allows for a thorough evaluation of the epithelial architecture of the lesion [35]. For OPMDs such as OLP, current practice is to recall the patients regularly and observe for any changes, such as loss of homogeneity, which may indicate carcinogenesis and to biopsy the lesion as indicated [36, 37].

However observation of any such change is highly clinician dependent and even with meticulous follow up, early malignant changes may be overlooked [38]. Specifically, poor practice of routine oral mucosa examination during dental and medical recalls is reported by almost all patients whose oral cancer was diagnosed at a late stage [39]. In addition, histological changes indicative of dysplasia can be found even in clinically normal mucosa [40, 41]. While screening programmes to identify malignant lesions have been trialled, their cost effectiveness in the general population is uncertain and the onus has fallen on primary care providers to screen patients for such lesions [42–45]. Currently, the US preventive task force states that there is insufficient evidence to assess the balance of benefits or harm of routine screening for oral cancer [46]. Of concern, a meta-analysis has indicated that a COE while having a relatively high sensitivity at 93 % has a poor specificity at 31 % and cannot reliably differentiate between benign and dysplastic lesions [40]. Analysis states that a number of benign conditions mimic oral malignancies and dysplasia may be found in clinically normal mucosa [40]. The review suggests that further research should be undertaken into adjunct technologies to improve the reliability of clinicians in screening for malignant and potentially malignant disorders [40]. These devices use the principles of vital staining, reflectance, tissue autofluorescence or optical molecular imaging and aim to enhance visual detection of lesions and to differentiate between benign and malignant lesions [3]. This concept is utilised in commercially available devices such as toluidine blue, ViziLite Plus™, Microlux/DL™, Orascoptic DK™, VELscope™, Identafi®, DOE SE Kit™, Sapphire Plus™ and ESPýOC [47–54]. The aims of these products are twofold: firstly to aid the practitioner in the detection of potentially malignant lesions, and secondly to highlight areas of clinically visible lesions which are most likely to have undergone dysplastic changes. This could assist in determining the ideal site of biopsy and also the lesion's margins to delineate the extent of excision required. This chapter discusses the current literature regarding the efficacy of these systems as diagnostic adjuncts for detecting malignant lesions or potentially malignant disorders.

9.2 Oral Potentially Malignant Disorders (OPMD)

9.2.1 Leukoplakia

Oral leukoplakia is defined by World Health Organization (WHO) as a 'a white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer' [55]. It is the most common OPMD. Using a pooled analysis, the estimated global prevalence of leukoplakia is approximately 2 % although this figure is likely to be an overestimation [32, 56]. Leukoplakia is six times more prevalent in smokers, and alcohol use is also a risk factor in its development [32].

Clinically, leukoplakia may be classified as either homogenous or non-homogenous. This distinction is based upon surface colour and morphological characteristics. A lesion is considered homogenous if it is flat, thin and uniform in colour [25, 32]. A non-homogenous leukoplakia contains red areas although remains predominantly white and may be speckled or nodular [25, 32]. Figure 9.1 shows an example of a slightly fissured homogeneous leukoplakia. A non-homogenous leukoplakia is associated with a greater risk of malignant transformation [25, 57, 58]. A change in clinical diagnosis from a homogenous to non-homogenous leukoplakia was associated with a 4.2 times increase in the risk of a histological diagnosis of dysplasia on biopsy [58].

While the malignant potential of oral leukoplakia is undisputed, the reported rate at which this occurs varies greatly. A hospital study in Netherlands found an annual transformation rate of 2.9 %, while a recent study in China reported a rate of 3.38 %, both of which are higher than previous estimates [57, 59]. In Australia, the annual

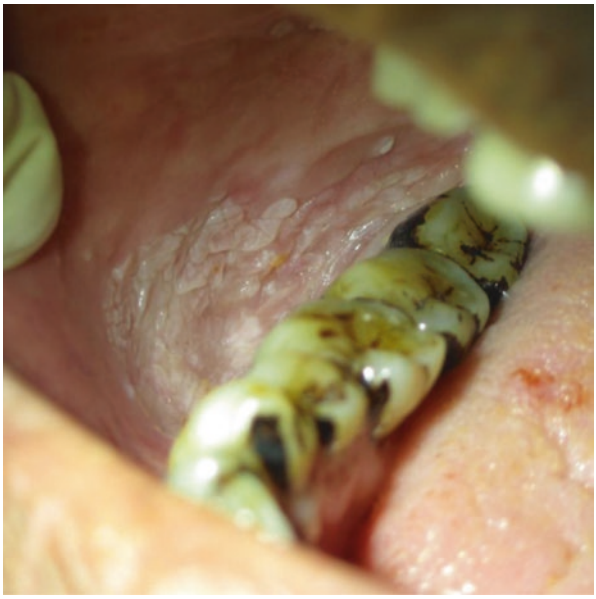

Fig. 9.1 Slightly fissured homogeneous leukoplakia in the right mandibular buccal vestibule of a patient. A biopsy confirmed the presence of moderate dysplasia in this patient

malignant transformation rate was calculated to be approximately 1% [58]. Interestingly, while smokers have a higher rate of leukoplakia in established lesions, there is a higher malignant transformation rate in non-smokers particularly among women [32, 57, 60]. Women in general seem to have a higher risk of malignant transformation of oral leukoplakia [57].

Currently, the only clinical predictor for the risk of malignant potential is whether or not the lesion is homogenous. Histologically, as defined by the World Health Organization (WHO), oral leukoplakia may be classified as hyperplasia, mild dysplasia, moderate dysplasia, severe dysplasia or carcinoma in situ [55]. A higher grade of dysplasia is associated with an increased risk of malignant potential [32, 57]. Liu et al. proposed a new approach to classifying lesions as either 'high risk' or 'low risk' according to the number of characteristics of epithelial dysplasia the lesion displayed on histological analysis [59]. A high risk lesion was one which displayed at least four architectural changes and five cytological changes on histological analysis. It was found that a high risk lesion had a 4.57 fold increased rate of malignant transformation. This approach provides a less arbitrary method of classification since it is known that there is a large amount of inter-operator variability in determining the degree of dysplasia using traditional methods.

9.2.2 Erythroplakia

Erythroplakia has been defined as a fiery red patch which cannot be characterised clinically or pathologically as any other definable disease [25, 32, 55]. The reported prevalence varies between 0.02 and 0.2% [61]. The lesion may be flat or depressed and is predominantly on the floor of mouth, soft palate, ventral tongue and tonsillar fauces [61]. Generally, the lesion is a solitary one which helps distinguish the condition from other systemic conditions [32]. While the condition is not as prevalent as leukoplakia, on biopsy it frequently demonstrates epithelial dysplasia. In one study, from 65 biopsies of erythroplakia, 51% displayed invasive squamous cell carcinoma while a further 40% were carcinoma in situ highlighting the seriousness of the condition [62]. Of those which display epithelial dysplasia, a large proportion of these undergo malignant transformation [32]. They may also present as mixed white/red lesions which are termed erythroleukoplakia (more commonly referred to as speckled leukoplakia) [25]. Figure 9.2 shows an example of an erythroleukoplakia lesion.

9.2.3 Oral Lichen Planus

Oral lichen planus is a T-cell mediated chronic inflammatory condition of the oral mucosa. It is characterised by a subepithelial lymphocytic infiltrate leading to degeneration of basal cell keratinocytes, although the stimulus for immune activation is currently unknown [63]. OLP has varying clinical presentations with the reticular, atrophic and erosive forms being the most common, although multiple

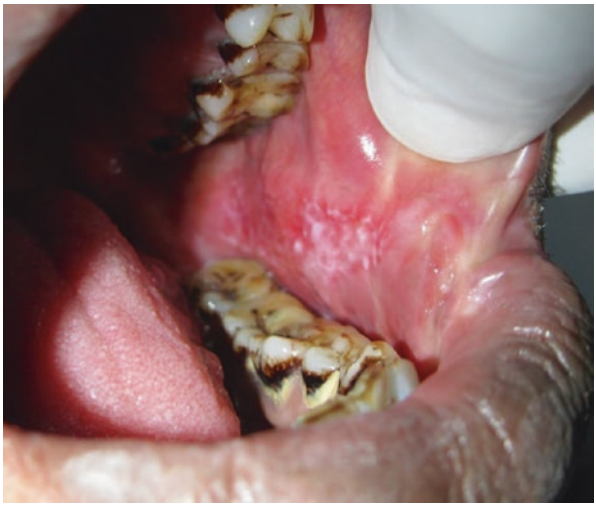

Fig. 9.2 Suspicious speckled leukoplakia lesion in the left buccal mucosa of a long-term tobacco user (smoking and chewing tobacco). A biopsy confirmed the presence of squamous cell carcinoma

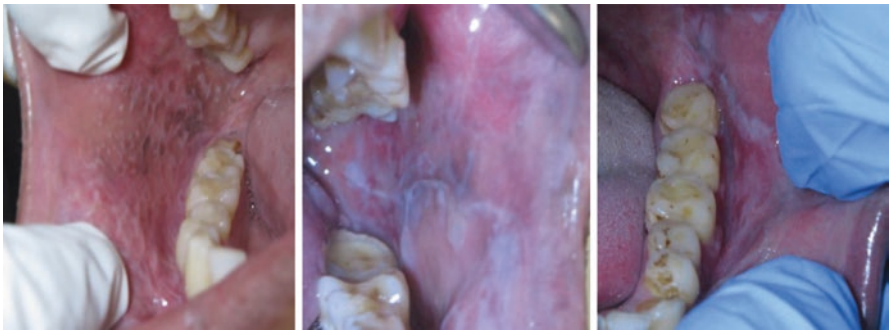

Fig. 9.3 Oral lichen planus (*OLP*) patient examples. *Left* and *Middle* – reticular form of OLP. *Right* – erosive form of OLP

forms may present concomitantly and clinical presentation of the lesion may change over time [64]. Figure 9.3 provides some example images of OLP. Lichen planus may also have extra-oral involvement with vaginal and cutaneous lesions being the most common [65, 66]. Typically, the condition presents with bilateral and symmetric lesions [65]. Symptoms may vary from completely asymptomatic to severe pain and discomfort which prevents proper intake of food. Factors such as stress, systemic illnesses, certain foods, poor oral hygiene or dental treatment may exacerbate the condition in some cases [64]. Currently, treatment for the condition is largely symptomatic through the use of topical corticosteroids to suppress inflammation. Asymptomatic cases generally do not require any management other than periodic follow-up.

Of much controversy is the malignant potential of OLP. Many authors have argued that a small percentage of cases of OLP progress to oral squamous cell carcinoma.

The reported rate at which this occurs varies from 0 to 10 %, although around 1 % is the commonly quoted figure [36, 65, 67–70]. It is known that chronic inflammation may lead to malignancy as seen with the development of colorectal cancer in inflammatory bowel diseases and oesophageal carcinoma after esophagitis among others [63]. This may be due to damage to DNA, disruption of tissue architecture and function via activation of stromal cells and components or due to changes in the extracellular matrix. An over-expression of p53 has also been noted in OLP lesions; however this may be a response to damage due to chronic inflammation [37].

Many authors have observed that OSCC arising in OLP have different behavioural characteristics to regular OSCC which suggests that molecular events leading to malignancy may differ from classical epithelial dysplasia [63]. OSCC arising in OLP has been found in patients without classical risk factors such as alcohol and smoking and seems to be more common in women [36, 37, 71]. There is reportedly a higher rate of secondary tumour formation in OSCC arising from OLP compared with oropharyngeal cancer without a previous diagnosis of OLP with Mignogna et al. finding at least one secondary tumour in 53 % of OSCCs arising from OLP [72]. These findings have lead Mignogna et al. to propose that OLP could cause field cancerisation of the oral mucosa [73]. It is theorised that the chronic inflammation may cause widespread changes in the oral mucosa increasing its propensity to develop OSCC. It is known that chronic inflammation can be seen histologically in clinically normal tissue around lichenoid lesions [74]. This may explain both the high rate of secondary tumours and also the development of OSCCs in oral mucosa distant to the primary OLP lesion. OSCC arising in lichen planus also displayed a greater tendency to metastasise with micro-invasive carcinomas of 1.75 mm displaying nodal involvement when generally this does not occur until greater than 4 mm of invasion has occurred [36, 75].

Due to the small risk of malignant change, current ideology is to periodically recall patients diagnosed with OLP to detect any malignant changes at an early stage. However the effectiveness of this protocol itself has been questioned [67, 76]. A significant problem exists in that there is no definitive method of knowing if malignant change has occurred without taking a biopsy of the lesion. Currently, a biopsy may be taken if there are visible changes, such as loss of homogeneity, which indicate progression to malignancy; however studies have found a little if any difference in presentation of lichenoid lesions which do and do not proceed to malignancy [36, 72]. Further, many OSCCs found on follow up of OLP have been in sites other than those which were initially biopsied which further highlights difficulties in identifying early malignant changes [65]. When comparing a follow-up period of 4 months and 12 months, there was no reduction in mortality from OSCC arising in patients with OLP although tumours were diagnosed at an earlier stage [36, 77]. In one publication, even a 4 month follow up with periodic biopsies resulted in a 50 % mortality rate among six patients who were diagnosed with OSCC on follow up [38]. This may be due to either failure to detect malignant changes or rapid progression of the lesion to malignancy.

Due to these findings and the low rate of malignant transformation, specialist follow up does not seem cost effective [76]. A large part of the failures of

screening seem to be the lack of definitive criteria on what to biopsy. Currently, it is largely clinician dependant and dysplastic changes may easily be overlooked. A similar situation can be seen with screening for gastric and colon cancers. When a new criterion was introduced for gastrointestinal endoscopic surveillance in western countries, the early diagnostic rates of these cancers increased [38]. Future areas of research may be to more easily discern and identify which areas of the mucosa are more likely to proceed to malignancy. Zhang et al. found that toluidine blue is more likely to stain dysplastic lesions which proceed to malignancy than those which do not [78]. This could prove to be of use if similar effects were found in lichenoid lesions.

While the argument of the malignant potential of OLP has for some time been controversial, this was further complicated by the proposition by Krutchkoff and Eisenberg of a distinct histopathological entity referred to as lichenoid dysplasia [79]. Krutchkoff claimed that cases of malignant transformation of OLP were initially cases of dysplasia with lichenoid inflammation which was misdiagnosed on initial histological analysis due to their similarities [80]. This was based on a review of reported cases of malignant transformation of OLP of which many, on retrospective analysis, displayed dysplasia on the initial biopsy. This separate entity was labelled lichenoid dysplasia; however, no consensus on the topic has been reached. It is unknown if this is a dysplastic change due to chronic inflammation which would represent true malignant transformation or if the dysplasia was always present and the diagnosis was complicated due to the presence of a subepithelial lymphocytic infiltrate. It is also possible that the inflammatory infiltrate is an immune response to the dysplastic changes if tumour cells are detected [81]. Much of the difficulty may arise due to the difficulty in histological diagnosis of OLP. It has been shown that if over-riding characteristics of lichen planus are present on a histological specimen, dysplasia may easily be overlooked and dysplastic characteristics may be disregarded as reactive changes to inflammation [82]. Further to this, OLP itself displays many features which are characteristic of epithelial dysplasia even when dysplasia is not present [82, 83]. Due to these similarities, there is a high level of inter-observer variability in the histological diagnosis of OLP and it is proposed more stringent criteria may be required [84].

Zhang et al. performed molecular analysis of OLP lesions to determine if there was a loss of heterozygosity (LOH) in these lesions which may indicate malignant changes in these lesions. It was found that LOH in lesions diagnosed as OLP is similar in LOH to reactive lesions rather than dysplasia indicating that oral lichen planus itself does not have a malignant risk [85, 86]. However, a similar analysis in lesions diagnosed as lichenoid dysplasia found similarities in LOH to epithelial dysplasia giving further weight to the argument that it is lichenoid dysplasia, not OLP itself which has a malignant potential.[87].

With the available evidence, the malignant potential of oral lichen planus still remains unclear. It is unknown if OLP itself has a propensity to develop into OSCC or if there is indeed a separate entity referred to as lichenoid dysplasia. Since with current techniques it is impossible to predict which patients with OLP will develop OSCC, and due to the aggressive nature of OSCC arising in this cohort, the safest

option seems to be to review cases periodically to maximise the chance of detecting malignant changes at an early stage. The availability of histological features or molecular markers highlighting those OLP lesions which are at a high risk of malignant change would be of great benefit as this could allow for targeted follow up.

9.2.4 Chronic Hyperplastic Candidosis

Candida is a commensal organism found in the oral microflora of 40 % of the population with *Candida albicans* being the most common among these [30]. While in the majority of cases they do not cause harm in the correct environment, such as if the patient is immunosuppressed they may become pathogenic. There have also been suggestions that candidal infection may predispose to malignant transformation of the oral epithelium, particularly in cases of chronic hyperplastic candidiasis. Chronic hyperplastic candidosis typically presents as 'an adherent chronic white patch on the commissures of the oral mucosa' [88].

It has been shown that in patients with epithelial dysplasia, the degree of dysplasia correlates with higher amount of yeast in the oral cavity [30]. Another study found an association between histologically confirmed fungal infection and epithelial dysplasia [89]. This study also found that among patients diagnosed with epithelial dysplasia, 21.9 % of lesions with a concurrent fungal infection progressed in the severity of dysplasia compared with 7.6 % of those without fungal elements [89]. Nagy et al. found in a cohort of 21 patients with OSCC that eight of these had candidal infection on the tumour site itself while none of them presented with candidal infection on a control site [90]. It is estimated that candidal leukoplakias progress to malignancy in 9–40 % of cases [91].

The reason for malignant transformation of the oral mucosa hasn't yet been determined. It is possible that *Candida* itself may produce carcinogens which lead to point mutations in the epithelium [88]. Certain strains of *C. albicans* have been shown to be able to convert ethanol into acetylaldehyde, an established carcinogen [92]. Localised increases in concentration of acetylaldehyde may initiate malignant changes in the oral epithelium. It is also possible that *C. albicans* does not initiate or exacerbate the malignant process but simply that dysplastic epithelium provides a favourable environment for the species to grow in. While the link between chronic hyperplastic candidosis and oral malignancy has not been established, an association between the two seems likely and further research is required.

9.3 Screening for Oral Cancer and Oral Potentially Malignant Disorders

The implementation of screening programs and standardised techniques has had a profound impact on the early detection and treatment of cervical cancer (Pap smears [93]) and breast cancer (mammography [94]). A universal standardised technique is needed for the oral cavity to help promote early detection and downstage the disease.

A thorough mucosal examination is currently recommended as part of a routine dental examination. Conventional oral examination (COE) has been shown to have high discriminatory ability [95] and is the currently accepted practice for the detection of oral cancer and OPMD. Detection of lesions may be enhanced by the use of adjunctive aids such as toluidine blue, diffused white light, chemiluminescence or loss of tissue autofluorescence [2]. As research continues into the efficacy of these adjuncts, an important consideration though is how factors such as the experience and confidence of the practitioner and the acceptance of the patient of the procedures influence their effectiveness.

Oral cancer screening has been defined as 'the process by which a practitioner evaluates an asymptomatic patient to determine if he or she is likely or unlikely to have a potentially-malignant or malignant lesion' [3]. This may occur as 'population based screening', when a population is assessed specifically for the purpose of detecting oral cancer; as 'opportunistic screening', when patients who are attending a health care provider for another purpose are examined for signs of oral cancer or OPMD; or as 'targeted screening', when high risk individuals are selected for screening [44]. In any of these contexts, along with a visual and tactile examination of the oral mucosa, the practitioner should ask the patient about their health history including tobacco and alcohol use. The risk of oral cancer is increased with age, alcohol and tobacco use and a history of upper aerodigestive tract cancer. The term 'oral cancer screening' should include an oral mucosal examination together with an assessment of the individual's health history, including symptoms and risk factors. Clearly, oral cancer screening is only one component of a comprehensive 'oral examination'.

A 2010 Cochrane review [44] assessed the effectiveness of current screening methods in reducing oral cancer. Only one study met the inclusion criteria. This study commenced in Kerala, India, in 1995 and involved over 190,000 participants in 13 clusters [45, 96]. The Kerala RCT was a population-based screening program and is the single randomised controlled trial conducted to date. This trial demonstrated a stage shift such that cases were identified at an earlier stage in the screened group compared with the control group. Significant methodological limitations leading to a high risk of bias were identified by the Cochrane review with the design of this RCT. These limitations included lack of detail regarding random assignment of clusters, small number of clusters, no analysis of the effect of clustering on the results, no blinding of the outcome assessment and lack of information about withdrawals and drop-outs. In addition, only 63 % of participants with positive screen results complied with referral and a low proportion of lesions were biopsied for histological confirmation of diagnosis. The authors of the review concluded that while there was some evidence from the single included study that visual examination as part of a population-based screening programme reduced oral cancer mortality for high risk individuals, further well-designed RCTs were needed. While RCTs represent the highest level of evidence for assessing the efficacy of an intervention, whether this type of study design is the most appropriate way to determine the usefulness of screening for oral cancer is debatable. The lack of rigorous trials is likely due to feasibility and cost issues related to the very large sample sizes required

because of the relatively low incidence of oral cancer in the general population [97]. Using diagnosis of oral cancer (cancer registry data) as the outcome measure requires a long duration of follow up (the Kerala trial used a 9 year follow up) so that data can be collected from a control group. It also assumes that cancer registry data is complete and accurate. An RCT study design clearly also raises ethical issues of withholding the screening from control participants.

A free screening clinic in the USA reported that suspicious lesions were found in 5 % of patients, and 1 % of patients were confirmed to have oral cancer or OPMD [98]. The authors concluded that due to the low prevalence of oral cancer, screening should be targeted to high-risk groups. A surprising finding from this study was that half the patients with confirmed malignancies did not return for follow-up treatment. So while population-based screening cannot currently be recommended due to lack of available evidence, further research should focus on targeting high risk groups. In addition, it has been recommended that research to explore the psychosocial factors influencing outcomes of screening programmes and patient experience and understanding of cancer diagnosis be undertaken [99].

The objective of early detection in oral cancer is to recognise not only oral cancer but OPMD at the earliest possible stage. Referral of these lesions to a specialist will result in an early definitive diagnosis and treatment if indicated. Even though accurately predicting malignant transformation for OPMDs displaying dysplasia is not currently possible, these lesions require special attention and particular management strategies depending on the site, grade of dysplasia and patient risk. The value of screening programs may not be solely limited to the detection of oral cancer. Screening opportunities should also be utilised to improve patient awareness about the relationship between risk factors such as alcohol and tobacco and oral cancer, which may play a role in prevention [100].

Populations at high risk of developing oral cancer are predominantly older, male, heavy users of alcohol and tobacco, and have a poor diet and low socioeconomic status [101]. Since the prevalence of disease is higher in these groups, opportunistic screening programmes targeted to these populations may have greater effectiveness [44]. The cost effectiveness of this approach has been supported by the results of a study using a simulation model [102] as well as the Kerala trial [103]. An increase in the incidence of HPV-related oral cancers however means that the demographics of the high risk patient are changing and dichotomising, as these lesions are diagnosed at younger ages than HPV-unrelated oral cancers [104]. An additional risk factor particular to South Asian cultures is the chewing of areca nut and betel quid (with or without tobacco). These products are inexpensive and addictive and their use is widespread and starts at an early age. People from these cultures, either residents in their home country or migrants to other countries, also represent a high risk group for whom screening programmes may be effective. There are however social and cultural factors (religion, perceived health benefits, first or second generation immigrants) which influence the use of these products and the risk of oral cancer so these factors should be further investigated.

Other racial groups including African-Americans [105], Hispanics in New York [106] and Indigenous Australians [107] have also been shown to have a higher prevalence of oral cancer and this is probably largely due to increased smoking and

alcohol use and lower socioeconomic status of these groups. In the case of Indigenous Australians however there is conflicting data present [108]. Farah and colleagues have recently documented the oral mucosal burden in an urban Indigenous community in a general dental practice. The urban Indigenous community assessed did not display significantly higher rates of smoking, alcohol consumption or oral mucosal lesion prevalence compared to non-Indigenous counterparts [108].

People in developing countries have higher rates of oral cancer and this has been suggested to be due to greater exposure to risk factors and from an earlier age [109]. Low socioeconomic status itself however has been significantly associated with oral cancer risk in both developing and developed countries and this association remained after adjusting for known risk factors (alcohol, smoking, diet low in fresh fruits and vegetables and HPV infection) status [101]. While the reasons for this are not yet fully understood, low levels of education and income are likely to affect access to health care, nutrition, living and working conditions and life chances resulting in poorer health generally. There has also been a suggestion that the stresses associated with deprivation may alter the molecular biology of cancer and this also requires further investigation [101].

Recently the US Preventive Services Task Force (USPSTF) updated its 2004 recommendation on screening for oral cancer [110]. The USPSTF concluded that the current evidence was insufficient to assess the balance of benefits and harms of screening for oral cancer in asymptomatic adults. The revised guidelines apply to screening of the oral cavity performed by primary care providers, and not by dental providers or otolaryngologists. The USPSTF reviewed the evidence both on whether screening for oral cancer was associated with lower morbidity or mortality and on the accuracy of the oral screening examination to detect oral cancer or potentially malignant disorders that are highly likely to progress to oral cancer. The USPSTF found inadequate evidence that the oral screening examination accurately detects oral cancer or that screening for oral cancer and treatment of screen-detected oral cancer reduces morbidity or mortality. Furthermore, they found inadequate evidence on the harms of screening, as no study reported on harms from the screening test, from false-negative results or from false-positive results leading to unnecessary surgery, radiation and chemotherapy.

Similar to the USPSTF, the American Academy of Family Physicians concluded that current evidence is insufficient to weigh the balance of benefits and harms of screening for oral cancer in asymptomatic adults [110]. However, the American Cancer Society recommends that adults at least 20 years of age who have periodic health examinations should have the oral cavity examined as part of a cancer-related check-up [110]. The American Dental Association however recommends that practitioners remain vigilant during routine oral examinations for signs of potentially malignant lesions or early-stage cancer, particularly for patients who use tobacco or have heavy alcohol consumption [3].

With this in mind, recent data however suggests that dentists value the importance of oral mucosal screening, but that improvements in oral mucosal pathology education, with a focus on oral cancer prevention and detection are required [111]. There is also a need for change in undergraduate/graduate dental programmes to improve on communication skills of recently graduated dentists at least in the

Australian context where this study was undertaken, but it is very likely the same issues exist elsewhere. It has been recommended that competency in performing a full head and neck cancer screening and risk assessment should be included in graduate dentist recommendations in a fashion similar to that stipulated in the United States by the Commission on Dental Accreditation (CODA) [112, 113].

9.4 Detection of Oral Cancer and Oral Potentially Malignant Lesions

Early detection of oral lesions is based on the concept of clinically identifiable, OPMDs with increased risk of cancerous change [25, 32] preceding the development of most OSCCs and is the most effective method for improving patient survival and decreasing patient mortality [114]. Early identification of OPMDs is hindered by the clinical subtlety associated with these lesions [115]; however lesions of a non-homogeneous clinical appearance have been strongly associated with underlying oral epithelial dysplasia (OED) [33].

The current standard for detection of OPMDs and OSCCs is a conventional oral examination (COE), involving visual inspection and digital palpation of the oral cavity using incandescent light. However, this technique displays poor sensitivity for the detection of OPMDs [40, 42, 116–118] and is incapable of differentiating between progressive and non-progressive lesions [119]. These limitations have driven the development of new technologies designed to aid clinicians in detecting OSCCs and OPMDs with high sensitivity and specificity [120].

COE is the standard of care for detection of OPMDs and OSCCs but there are still significant limitations to this technique [40, 42, 116–118]. This has fuelled the search for adjunctive techniques to improve the efficacy for detection of these lesions and hence improve patient prognosis.

Epidemiological studies evaluating the prevalence of disease have historically used ‘screening’ interchangeably with ‘case finding’ despite the different implications of each term. Screening has been defined as: *the application of a test or tests to people who are apparently free from the disease in question in order to sort out those who probably have the disease from those who probably do not* [121]. Conversely, case finding refers to application of a diagnostic test or method to patients with abnormal signs or symptoms in order to establish a diagnosis. As such, case finding does not include assessment of symptom-free patients whereas the inclusion of these patients forms an integral part of screening [119]. In a dental setting, screening for oral squamous cell carcinoma (OSCC) and oral potentially malignant disorders (OPMD) occurs when patients report for care and is referred to as ‘opportunistic screening’[3].

9.4.1 Conventional Oral Examination

Conventional oral examination (COE) is the principal strategy used for the detection of oral mucosal changes, including identification of OPMD and OSCC, and

involves visual assessment the oral cavity with the aid of normal operatory incandescent or white light [119]. Digital palpation is utilised to assess oral tissues for clinical signs associated with malignancy such as induration and fixation [31].

Several studies have assessed the efficacy of COE performed by general practitioners in screening for mucosal changes and for differentiating malignancies from benign lesions. These studies included verification of the results of generalist examination by an oral medicine specialist which served as a 'soft' gold standard. The sensitivity and specificity findings ranged from 60 to 70 % and 95 to 99 %, respectively [42, 117, 118]. Downer and Epstein have also published systematic reviews assessing the effectiveness of COE in detecting and predicting a histologic diagnosis of an OPMD or OSCC [40, 116]. Downer determined 85 % sensitivity and 97 % specificity while Epstein reached a similar result for sensitivity, calculated at 93 %, but significantly poorer specificity for COE in detecting OPMDs and OSCCs – only 31 %. Epstein concluded that adjuncts are required to aid in detection and diagnosis of oral mucosal pathology as COE alone is not predictive of the histological status of oral mucosal lesions [40].

A recent Cochrane review identified only one randomised control trial out of 1719 articles evaluating screening for OPMDs or OSCCs using visual examination, toluidine blue, OralCDx brush biopsy or tissue autofluorescence imaging [44]. The cluster-randomised controlled trial by Sankaranarayanan et al. assessed the efficacy of COE in reducing the mortality rate due to OeSCC in Kerala, India [45]. The control group received routine awareness messages and were advised to visit their local health care centre. The intervention group received three rounds of oral visual inspection by non-medically trained health care workers every 3 years. Participants in the intervention group identified as having suspicious lesions were referred for diagnosis and treatment. Two hundred and five OSCCs and 77 OSCC related deaths were recorded in the intervention group with a mortality rate of 16.4 per 100,000 compared to 158 OSCCs and 87 OSCC related deaths in the control group which had a mortality rate of 20.7 per 100,000. The intervention group recorded a 5-year survival rate of 50 % while the control group recorded 34 %. There was also a 34 % reduction in the mortality rate noted in the tobacco and alcohol users group indicating that COE is effective at reducing the mortality rate associated with OSCCs in these high risk patients.

COE is the mainstay of early detection for general practitioners but is incapable of identifying all OPMDs and OSCCs [40, 42, 116–118] and cannot differentiate between progressive and non-progressive lesions [119]. Although oral specialists are typically better trained at recognising subtle changes associated with early carcinogenesis, clinical inspection alone cannot predictably differentiate between potentially precancerous, cancerous and benign lesions. Additionally, the oral cavity commonly presents with many types of benign lesions, which act to confuse health care professionals as they often have clinical presentations similar to precancerous and cancerous lesions (keratinisation, ulceration, inflammation, etc.). This has driven advances in technologies developed to serve as adjuncts to COE to improve the sensitivity and specificity for detection of OPMDs and OSCCs.

9.4.2 Diagnostic Adjuncts

9.4.2.1 Toluidine Blue

Tolonium chloride, more commonly known as toluidine blue, is a metachromatic, acidophilic dye which selectively stains acidic tissue components such as nucleic acids [122]. It has been used for decades as an adjunctive technique for diagnosis of OSCCs as well as to delineate the margins and extension of lesions more effectively [123].

Toluidine blue (TBlue) has been proposed as a tool for delineating malignant and potentially malignant lesions of the cervix and subsequently the oral cavity since the 1960s. Toluidine blue is a metachromic stain with affinity for DNA and RNA [124]. Areas of epithelial dysplasia contain a greater amount of DNA and RNA than normal tissue which correlates with an increased uptake of toluidine blue [124]. Furthermore, cancer by its nature of unregulated cell proliferation results in alterations in cell density [125, 126], which in essence leads to alterations in nucleic acid content per unit volume of tissue. The dye is available as a mouth rinse to find any lesion in the oral cavity or as a swab which can allow visualisation of specific areas [127]. *In vivo*, uptake of blue stain is associated with dysplastic tissue while lack of stain uptake is associated with benign lesions or normal mucosa [123]. Studies have reported partial, equivocal or a speckled pattern of stain uptake and also identified varying intensities of stain uptake and variably classify these as positive or negative [128]. While extensive research on the use of toluidine blue for identifying OPMDs is available, opinion on its efficacy and justification for routine use is divided among experts.

Initially, toluidine blue was assessed for its efficacy in highlighting OSCC. Multiple studies have found a high efficacy of toluidine blue in detecting OSCCs reporting 100% uptake of the dye [124, 127, 129–131]. This provides strong support for routine use of toluidine blue in specialist practice however ideally these lesions would be detected before invasion has occurred. In cases of OSCC seen in specialist clinics, staining characteristics are unlikely to alter the decision to biopsy therefore more consideration needs to be given to toluidine blue's performance in delineating dysplasia rather than its efficacy in highlighting OSCC. Margin studies for resection of OSCC have found that while toluidine blue will stain the tumour centre, it failed to identify the presence of dysplasia and carcinoma-in-situ on resection margins [124].

A number of groups have assessed the efficacy of toluidine blue for detecting OED in patients referred to specialist centres. Significant variation in accuracy of the technique is seen with reported sensitivities ranging from 50 to 100% [127, 129, 130, 132–135] and specificities ranging from 30 to 79% [127, 129–135]. Warnakulasuriya and Johnson reported the detection of five additional areas of dysplasia in 102 patients using OraScan™, a commercially available toluidine blue mouth rinse supporting its use in specialist centres [130]. The large variation in reported sensitivities may be due to the patient population and lesion exclusion criteria as inclusion of a large number of invasive carcinomas may increase the reported sensitivities while inclusion of early dysplasia may decrease the sensitivity. Toluidine blue appears to have a higher rate of take up with increasing severity of dysplasia which can be considered to be 'high risk' and a large number of false

negatives may be attributed to earlier stages of dysplasia [127, 135]. However, mild and moderate dysplasia may also proceed to malignancy and should be considered significant pathology. If toluidine blue alone was used to determine lesions to biopsy, these areas may be overlooked. The relatively low specificity is also of concern and may be attributed to inflammatory lesions which can also have rapid cell division increasing dye uptake [135]. For this reason, it has been proposed that reviewing lesions which appear benign but with uptake of dye may allow for reduction in false positives [130, 135]. Currently, no literature is available which has assessed this hypothesis.

Molecular studies support the previous hypothesis that the uptake of toluidine blue is associated with 'high risk' lesions. Epstein et al. found that toluidine blue uptake was associated with higher risk molecular profiles and increased allelic loss [136]. This is in agreement with Zhang et al. who found lesions with increased rates of loss of heterozygosity in lesions which stained positively [78]. In addition to this, toluidine blue positive lesions were associated with greater rates of malignant transformation and also earlier transformation [78]. Further studies are required to determine the clinical significance of these findings.

Onofre and colleagues assessed the reliability of *in vivo* staining using a 1 % toluidine blue solution [133]. Clinically obvious cases of OSCC or lesions without risk of malignancy were excluded from the study. Biopsy sites were selected on the basis of clinical appearance with lesion areas retaining the stain favoured as biopsy sites, and the clinical judgement of the oral medicine specialist directing the biopsy location if this did not occur. Toluidine blue displayed 100 % sensitivity for detection of OSCC and 50 % sensitivity for detection of OED [133]. Warnakulasuriya et al. established a sensitivity of 78 % for the detection of OED using a similar technique [130].

Staining and subsequent stratification of histopathological samples has also been used to evaluate toluidine blue *in vitro* for detection of OED or OSCC [137] 92 % of confirmed cases of OSCC retained the stain, but only 56 % of lesions displaying OED were detected by toluidine blue. The low sensitivity noted could be attributed to the classification used in the study as no distinction was made between low, moderate or high levels of OED and it has been established that toluidine blue is more effective at detecting higher levels of OED [130, 133, 137]. Another *in vivo* study utilising resected specimens also found that toluidine blue preferentially stained areas displaying higher levels of dysplastic change [124].

Several publications have claimed that the staining characteristics displayed by toluidine blue are based on the presence of high risk molecular clones within epithelium, regardless of the dysplastic status of lesions [136, 138]. In response to this, Zhang et al. attempted to relate the toluidine blue status of 100 OPMDs to their outcome, histopathological features and molecular risk patterns [78]. A strong correlation was found between toluidine blue staining, risk factors for OSCC and progression of lesions as 12 out of the 15 lesions that later underwent malignant change retained the dye. Toluidine blue positive lesions also displayed an increased frequency of high risk molecular patterns associated with malignant change. Preferential staining was also noted in lesions featuring higher clinical risks for malignancy due to location, size or nonhomogeneous appearance.

Toluidine blue has a high sensitivity for detection of frank carcinomas or severe OED but displays poor efficacy for identification of low to moderate OED [124, 130, 133, 137, 139, 140]. Therefore its clinical applications should be limited to identification of abnormal areas of lesions as suitable biopsy sites and supporting the decision to biopsy, not for visualisation of OPMDs.

Thus far, only one study has been conducted to assess the ability of toluidine blue to detect dysplastic lesions in general practice. Patients were invited to a mass screening programme aimed at detecting five neoplasms prevalent in the local community. Of about 7957 subjects participated in the toluidine blue arm of the trial, 4080 in the experimental group and 3895 in the control group using a placebo dye [141]. Those without high risk factors for oral carcinoma such as alcohol and betel quid were excluded from this study. Overall, two cases of oral cancer were ascertained in the experimental group and three in the control group. There was no significant difference in the detection of OMPDs between the two groups. Further, a 5-year follow-up of participants in the National Cancer Registry and National Household Registry found an additional three cases of oral cancer in both groups which suggests that screening with toluidine blue did not increase the rate of oral cancer detection. While this trial was conducted on those with high risk habits for oral cancer limiting the generalisability for a dental practice, this is likely to be the target population for potential oral cancer screening programmes. Although the 5-year follow-up may not be enough to detect a reduction in the incidence or mortality rates for oral cancer, the ultimate aim of cancer screening programmes, this initial data indicates no advantage of a toluidine blue rinse over the placebo dye in the detection of potentially malignant lesions.

Overall, toluidine blue seems to be an effective aid in delineating invasive carcinomas but not for the detection of potentially malignant lesions. Current evidence suggests it may be useful in a specialist care environment to help raise suspicion of lesions which may not otherwise be investigated or in the follow up of patients with a history of oropharyngeal cancer [124, 129, 133, 134]. Although toluidine blue does not accurately stain dysplasia, especially at its early stages, molecular analysis has suggested that lesions which retain toluidine blue are more likely to contain high risk molecular profiles and are more likely to progress to malignancy [78, 136]. This warrants further research in establishing the use of toluidine blue for predicting the risk of malignant transformation of established lesions referred for specialist care; however, currently the evidence does not support the use of toluidine blue in screening for OPMDs in the general population.

9.4.2.2 Reflectance Visualisation

There are two commercially available devices which utilise reflectance visualisation in conjunction with an acetic acid mouthwash to improve detection of OPMDs, and these include Vizilite™ (Zila Inc., CO, USA) and Microlux/DL™ (AdDent, Danbury, CT, USA).

ViziLite™ and ViziLite Plus™

ViziLite™ (Zila Inc., CO, USA) is a commercially available kit utilising the principles of chemiluminescence to enhance the visibility and detection of OPMDs.

Like toluidine blue, the principles on which ViziLite™ are based are adopted from similar technologies used in the detection of cervical cancer. The system utilises a 1 % acetic acid mouth rinse to desiccate the oral mucosa followed by visualisation of the oral cavity under a chemiluminescent light source. ViziLite™ utilises a chemiluminescent light stick containing an inner glass vial of hydrogen peroxide and an outer plastic capsule of acetyl salicylic acid to assess the oral cavity [48, 142]. The updated version, ViziLite Plus™, uses the same chemiluminescent system but recommends follow up of 'aceto-white' lesions with TBlue, a toluidine blue swab, to further visualise the lesion [49]. To use, the ViziLite™ light stick is bent breaking the glass inside and resulting in chemiluminescence emitting a bluish-white light with a wavelength ranging from 430 to 580 nm [143]. The manufacturer claims that following the application of acetic acid wash, mucosal abnormalities are better visualised due to changes in their refractile properties and are seen as 'aceto-white' as compared to normal epithelium which appears as a blue hue [49].

Currently, evaluation of ViziLite™ has been limited to specialist centres. A number of authors have reported that ViziLite™ enhanced visualisation of lesions based on characteristics such as ease of visibility, texture, brightness or sharpness of lesions [48, 142, 144–147]. However, Oh and Laskin believe that the use of ViziLite™ hindered visualisation of lesions, while acetic acid mouthwash itself without the addition of chemiluminescence may aid lesion visualisation [148].

While ViziLite™ has not been marketed as a diagnostic aid, but rather designed to help visualisation, studies assessing its efficacy in detecting dysplasia and OSCC have reported sensitivities between 77.1 and 100 % and specificities from 0 to 30 % [48, 142, 149, 150]. Conversely, Mehrothra et al. found four cases of dysplasia among 102 patients all presented negative using ViziLite™ [151]. Concerns with the ViziLite™ system are that almost any white lesion appears as a positive and a number of benign conditions such as leukoedema or hyperkeratosis are also likely to appear 'aceto-white'[48, 152]. With this in consideration, ViziLite™ doesn't seem to provide any benefits in differentiating OPMDs from benign lesions since any white area can appear positive. Further, it is reported that red lesions are less likely to be detected under ViziLite™ than with incandescent light alone [144, 146]. This is a significant finding considering that erythroplakia is highly associated with dysplasia and these may be missed using ViziLite™ alone for lesion detection. Rajmohan et al. stated that ViziLite™ was effective in delineating precancer or cancer which presented as either keratotic or mixed red-white lesions however failed to detect lesions, including an OSCC, which presented as erosive lesions [153].

Farah and colleagues used ViziLite™ in 2006 to examine 55 patients referred for the assessment of leukoplakic oral mucosal lesions in a specialist oral medicine setting [48]. Details such as lesion size, location, ease of visibility and the presence of satellite lesions were recorded during an initial COE. After rinsing with the 1 % acetic acid solution, the measurements were repeated using ViziLite chemiluminescent illumination. All lesions were biopsied, and the presence of OED was considered a positive finding. ViziLite™ had no effect on the border distinctness of lesions, lesion size or the choice of biopsy site. However, there was one instance where the device aided in the identification of a satellite lesion that was not detected by

COE. ViziLite™ could not distinguish between malignant and benign lesions as all lesions appeared 'aceto-white' and were considered to be ViziLite™ positive. The device displayed a specificity of 0 %, sensitivity of 100 % and 18 % accuracy.

The poor accuracy observed by Farah et al. was not noted in another study, examining a high risk population, which recorded 81 % accuracy for ViziLite™ in addition to sensitivity and specificity of 100 % and 14 %, respectively [142]. Only 75 % of the original lesions were biopsied; therefore the accuracy of these results cannot be determined and this study was given a low quality rating by a subsequent review [128].

Epstein and colleagues assessed 84 patients using ViziLite™ in 2008 [145], and this study methodology was adopted by Mojsa et al. in 2011 [147]. Epstein identified 97 lesions in 84 patients while Mojsa et al. used ViziLite™ to assess 30 patients with 41 lesions suspected of being premalignant. Both studies reported increased brightness or sharpness of lesions in 58–62 % of cases. ViziLite™ was only used on lesions visible during COE and both studies noted that sensitivity and specificity were not significant as negative results on initial COE were excluded on ethical grounds. Epstein noted 76 false positive (FP) and 20 true positive (TP) findings using ViziLite™, while Mojsa recorded 5 FP and 19 TP findings.

Vizilite™ has also been utilised to assess erythroplakic and erythroleukoplakic lesions in a study by Awan and colleagues [149]. The device demonstrated reduced efficacy for the detection of lesions of this type compared to leukoplakic lesions [48, 142], with sensitivity and specificity findings of 77 and 27 % respectively. Only 75 % of the 126 lesions assessed in this study were detected, enhanced or appeared aceto-white during examination using the ViziLite™ system. This study was the first to acknowledge that use of the acetic acid mouthwash increased salivary flow, which enhanced mucosal reflectance and hindered delineation of lesion margins. As expected, Awan et al. concluded that ViziLite™ could not discriminate between low and high risk lesions and as such vigilant interpretation of the results of ViziLite™ examination is required [149].

ViziLite™ has also been assessed in two opportunistic screening studies which utilised OralCDx brush biopsy for the evaluation of lesions which could not be diagnosed clinically [148, 152]. Following initial COE, Oh and colleagues re-examined patients using incandescent light and the acetic acid solution followed by examination using both the solution and the light component of the ViziLite™ system [148]. The lesions detected at each stage were noted as well as whether the lesions could be diagnosed clinically. Ninety-five lesions were detected in the study, 83 during COE and the rest using incandescent light and the acetic acid rinse. Thirty-two lesions could not be diagnosed clinically and were submitted to OralCDx and of these only two returned atypical results but proved to be benign following scalpel biopsy.

Huber et al. conducted COE followed by examination using the complete ViziLite™ system [152]. One hundred and forty-two cases of linea alba and leukoedema were accentuated by ViziLite™ and were diagnosed clinically without the requirement for biopsy. Two out of 14 lesions clinically diagnosed as frictional irritation were amplified by ViziLite™ and the provisional diagnoses of all lesions

were confirmed via OralCDx. The authors noted one instance of an amplified lesion in a high risk patient which displayed cellular atypia following scalpel biopsy as well as another lesion only detected by ViziLite™ which returned a result of mild atypia following evaluation with OralCDx.

Finally, subjective comparisons of lesion size, discreteness and sharpness have been used to assess the efficacy of ViziLite™ in visualising oral mucosal lesions [144, 146]. Epstein and colleagues conducted COE and examinations using ViziLite™ on patients with a history of mucosal lesions or newly detected lesions [144]. ViziLite™ failed to detect three lesions, and of these two were erythroplakic but not suspicious for malignancy, and histology confirmed the third as oral lichen planus. Two lesions were only visible following ViziLite™ examination but only one was biopsied and confirmed to be recurrent OSCC, the second was determined clinically to be benign. The device improved the brightness of 54 % of lesions, 40 % had more distinct borders and 36 % had a more defined texture when using the device. An increase in size was noted in 15 % of lesions although the difference in lesion size was not statistically significant.

Kerr and colleagues conducted COE followed by ViziLite™ examination on 501 high risk patients [146]. One hundred and twenty-seven lesions were classified as suspicious and of these 61 % were visually enhanced by ViziLite™, compared to 6 % of 363 non-suspicious lesions. The study established that the appearance, size, location and the type of lesion determined if it were be detected by ViziLite™, with red lesions the least likely to be detected. There were six aceto-white lesions initially detected only by ViziLite™ but visible upon re-examination using COE; however, these lesions were not biopsied and their meaning is ambiguous. As expected, the study concluded that ViziLite™ resulted in a significant increase in the sharpness of lesions but differences in lesion brightness and texture were not statistically significant.

The literature indicates that ViziLite™ cannot differentiate between malignant, premalignant lesions or benign lesions [48, 149] but is capable of improving the visibility of lesions [144–147]. Studies involving larger cohorts of participants and with uniform histopathological correlation are required before the device can be recommended as an adjunctive method for early detection.

Using Vizilite Plus™, Epstein et al. found all serious pathololgy was more easily visualised [145]. The authors found that among 97 lesions, toluidine blue tested positive for all 20 cases of severe pathology however only included OSCCs, carcinoma-in-situ or severe dysplasia as severe pathology. In cases of mild and moderate dysplasia, 59 % of cases stained positive with toluidine blue. The authors argue using this system would result in a 55 % reduction in biopsies by only biopsying lesions which retain the dye. However, using this protocol a number of lesions with mild and moderate dysplasia would be missed. Considering that mild and moderate dysplasia can also progress to malignancy, these should be considered significant pathology. Kämmerer et al. found that while toluidine blue reduced false positives without increasing false negatives, there is a little clinical evidence to justify the additional cost of the system for diagnosis of suspicious lesions [150].

Current evidence indicates that ViziLite™ does not enhance the early detection of OPMDs or OSCCs. While lesion visualisation may be enhanced using ViziLite™, it is unlikely to alter the decision to biopsy or result in an increase in the detection of OPMDs [48, 142]. Further, the low specificity of ViziLite™ is likely to be exemplified in general dental practice where the prevalence of OPMDs is much lower than a specialist centre. In addition, ViziLite™ only aids the visualisation of white lesions while erythroplakic or erosive lesions may be missed [146, 149, 153]. Considering the low accuracy of toluidine blue as previously discussed, its addition is unlikely to be of any benefit. Current literature does not support the use of ViziLite™ or ViziLite Plus™ in the detection of OPMDs or OSCCs.

Microlux/DL™

Microlux/DL™ (AdDent, Inc. USA) is a light-based system sharing the same principles as ViziLite™. Instead of chemiluminescence, Microlux/DL™ emits a white light through an LED transilluminator. Like ViziLite™, the manufacturer recommends the use of a 1 % acetic acid rinse before use to alter the refractile properties of the mucosa after which leukoplakic lesions appear 'aceto-white' under Microlux/DL™ [154]. Currently only one clinical evaluation of Microlux/DL™ is available.

Farah and colleagues evaluated Microlux/DL™ on 50 patients referred to an oral medicine specialist for assessment of an oral lesion [47]. Following a conventional oral examination under incandescent light, patients were examined with Microlux/DL™ without the use of an acetic acid wash and then again following the mouth rinse. This was compared to an LED headlight which also employs white light and is commonly used by dental practitioners. Microlux/DL™ provided a sensitivity and specificity of 77.8 and 70.7 % when compared against a histopathological gold standard. The positive predictive value (PPV) was 37 % and negative predictive value (NPV) 94 % with 12 false positive (FP) and two false negative (FN) findings. The low positive predictive value indicates that Microlux/DL™ provides a poor indication of the underlying pathology of lesions and does not provide any additional benefit to oral examinations beyond improved lesion visibility and border distinctness.

Microlux/DL™ increased the visibility score of lesions from a mean of 3.28 for COE to 3.66 and produced an 81 % increase in the percentage of lesions with distinct borders. Microlux/DL™ also improved the visibility of lesions in 64 % of cases and the acetic acid component provided further enhancement in 12 % of cases; however the device had no effect on lesion size, choice of biopsy site or detection of satellite lesions. While lesion visibility was enhanced compared to a regular incandescent light, Microlux/DL™ did not uncover any new lesions or alter the provisional diagnosis or biopsy site. It was also poor in discriminating between benign lesions and OPMDs or oral malignancies. The LED headlight displayed similar properties to Microlux/DL™ in increasing lesion visibility and allowed for a greater field of view. The authors found that an LED head light provided further enhanced visibility, border distinctness and a more intense white light than the Microlux/DL™, with and without the use of the acetic acid mouthwash [47].

It should be noted that despite strict manufacturer instructions regarding the use of the acetic acid mouth washes with both ViziLite™ and Microlux/DL™, Farah and colleagues found no evidence that rinsing with acetic acid enhanced visualisation of oral lesions on inspection with Microlux/DL™, and this is reportedly the same with the ViziLite™ system. This then begs the question as to the usefulness and cost effectiveness of incorporation of the acetic acid rinses with these systems.

The results of this study were the first to demonstrate that white light provides superior visibility of oral mucosal lesions compared to incandescent lighting and supports the use of a white light source during oral mucosal examination. With the evidence available, Microlux/DL™ cannot be advocated for use in the detection of OPMDs or OSCCs, however it does highlight that white light is beneficial compared to routine incandescent operatory lights for the detection of oral mucosal lesions.

9.4.2.3 Optical Fluorescence Imaging (Tissue Autofluorescence)

Autofluorescence is a phenomenon whereby an extrinsic light source is used to excite endogenous fluorophores causing the natural emission of light from these compounds. Endogenous fluorophores include certain amino acids, metabolic products and structural proteins, among others [155]. The excitation and emission wavelength varies greatly between fluorophores. Within oral mucosa, the most relevant fluorophores are nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD) and collagen. NADH and FAD are metabolic products and are believed to be responsible for autofluorescence of epithelium [156]. Stromal fluorescence has been associated with crosslinks within the collagen matrix [156]. Additionally, tissue autofluorescence is also influenced by tissue absorption and scattering properties as the incident and autofluorescence light passes through the oral epithelium and connective tissue. For example, cancer-induced angiogenesis or inflammatory-induced increases in blood flow leads to an increased presence of submucosal blood. Haemoglobin, present in blood, strongly absorbs the incident light and autofluorescent light. Autofluorescence may be utilised in both an *in vitro* and *in vivo* manner. Multiple oncological applications for *in vivo* fluorescence spectroscopy have previously been described [157].

Within cervical epithelium, using UV excitation Pavlova et al. found a decreased fluorescence within the stroma with epithelial dysplasia using fluorescence microscopy [158]. Drezek et al. performed confocal microscopy studies using excitation at 380 nm and 460 nm within cervical epithelium [159]. At 380 nm, the epithelium displayed an increase in fluorescence with epithelial dysplasia; however like the previous study, stromal fluorescence decreased [159]. With a 460 nm excitation source, stromal fluorescence was again shown to decrease with dysplasia but with no significant difference in epithelial fluorescence [159]. This property has been translated into direct clinical applications with the advent of direct tissue autofluorescence. Using wavelengths in the 375–440 nm spectrum, *in vivo* autofluorescence could differentiate between grades of cervical intraepithelial neoplasia (CIN) with a high degree of sensitivity but could not differentiate between inflammation and CIN [160]. This is because many inflammatory conditions show similar alterations in

endogenous fluorophore (i.e., metabolic products, structural proteins, etc.) levels as well as an increase in blood flow and cell influx. Excitation with a 355 nm wavelength has also been found to be highly sensitive [161].

Confocal microscopy analysis of the oral mucosa finds similar results to those in the cervix. Using UV excitation in the epithelium itself, autofluorescence increased slightly in dysplasia however was associated with a decrease in inflammatory lesions [156]. However, both dysplasia and inflammatory lesions were associated with a loss of stromal fluorescence using excitation with both UV and a 488 nm wavelength [156]. Using Monte-Carlo modelling, inflammation seems to show a greater loss of autofluorescence than dysplasia since it occurs at both the stromal and epithelial level [162]. Roblyer et al. demonstrated that UV-A tissue autofluorescence (365 nm) provided a sensitivity and specificity of 83.7 and 86.8 %, respectively, when delineating oral precancerous and cancerous lesions from normal tissue [163]. De Veld et al. obtained similar values (sensitivity 89 %, specificity 71 %) in a much larger study population of 172 patients with benign, dysplastic or cancerous lesions and 70 normal, healthy volunteers [157, 158, 162, 164]. Furthermore, Gillenwater et al. concluded that tissue excitation with 365 nm light was one of the wavelengths that provided the greatest ability to resolve oral cancer and normal tissues [165]. Other investigators have found using an excitation wavelength of 410 nm and emission wavelength of 635 nm can differentiate oral lesions with a high degree of sensitivity and specificity [166]. Commercial devices have become available for *in vivo* detection of epithelial dysplasia in the oral mucosa and these are described in greater detail below [120].

Assessment of mucosal autofluorescence properties involves illumination of the tissue using the visible light spectrum. This causes absorption of a portion of the photons by fluorophores such as NADH and FAD located in the epithelial layer [167], or elastin and collagen located in the stroma [155]. The fluorophores then emit lower energy photons which can be detected from the mucosal surface as fluorescence [167]. The presence of disease can alter the absorption properties of tissue due to changes in blood concentration or nuclear size distribution. Changes in epithelial thickness, such as epithelial hyperplasia, can limit the fluorescence signal produced by the strongly fluorescent collagen layer and these lesions will display loss of autofluorescence (LAF) [168]. Malignant lesions display reduced fluorescence due a reduction in the number of collagen and elastin crosslinks, as well as altered concentrations of FAD or imbalance between fluorescent NADH and non-fluorescent NAD+. In particular, several researchers have illustrated that oral cancer cells have lower levels of NADH due to aberrations in cell metabolism during carcinogenesis [169, 170]. Carcinogenesis affects the distribution and concentration of fluorophores within the epithelium and stroma and influences the ability of the epithelium to emit fluorescence after stimulation with an excitation light [156, 167, 168, 171, 172].

Preliminary research indicated that autofluorescence was a suitable adjunct to COE in early detection of OSCC and OPMD [163, 167, 172–174]. This technology has now been incorporated in two adjunctive devices aimed at early detection of OPMDs and OSCCs.

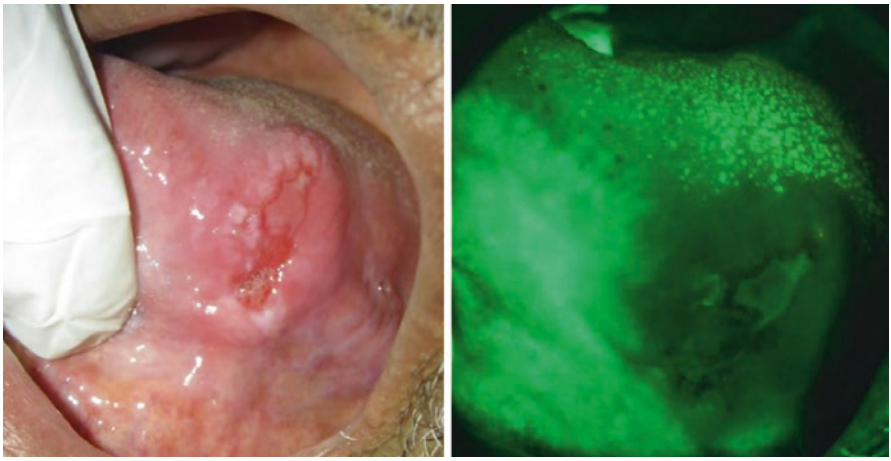

Fig. 9.4 Imaging of a biopsy confirmed squamous cell carcinoma lesion via standard white light (*left*) and through the VELscope Vx™ product (*right*). The lesion clearly demonstrates a loss of autofluorescence (*LAF*) when visualized through the VELscope Vx™ product

VELscope™

LED Medical Diagnostics Inc. (Vancouver, BC, Canada) in partnership with the British Columbia Cancer Agency developed VELscope™ (Visually Enhanced Lesion Scope), a system marketed as an extra-oral device that is intended to be used by a dentist or healthcare provider as an adjunct to traditional oral examination by incandescent light to enhance the visualisation of oral mucosal abnormalities that may not be apparent or visible to the unassisted eye. VELscope™ is further intended to be used by an oral surgeon to help identify diseased tissue around a clinically apparent lesion and thus aid in determining the appropriate margin for surgical excision.

VELscope™ (LED Medical Diagnostics Inc., Barnaby Canada) uses the principles of autofluorescence to differentiate benign mucosa from malignant and potentially malignant lesions [175]. VELscope™ utilises blue light excitation in the 400–460 nm wavelength [3, 168, 176, 177]. At this excitation wavelength, normal oral mucosa is associated with a pale green fluorescence while abnormal tissue is associated with a loss of autofluorescence (LAF) [3]. Pilot studies found that this excitation wavelength could be used *in vivo* to differentiate normal oral mucosa from dysplasia, carcinoma-in-situ and invasive carcinoma, and the manufacturer extrapolated these findings to claim that VELscope™ may be used to detect oral mucosal abnormalities not visible under white light examination [173, 175]. Figure 9.4 shows an example squamous cell carcinoma lesion under white light and visualised through the VELscope Vx™ (cordless handheld version of the VELscope™)

Early research supporting the use of VELscope™ was based on case reports conducted on patients referred to or on a follow-up programme at specialist oral dysplasia clinics [178, 179]. Kois et al. found VELscope™ assisted in the detection of dysplastic and malignant lesions not visible under COE and helped raise suspicion

of lesions which would otherwise not be subjected to biopsy [178]. For example, in one case where widespread erythema was present confounding the operator on which areas to investigate, VELscope™ revealed an area which later proved to be a well-differentiated carcinoma. It can also be a valuable tool in demarcating margins of established tumours where the malignant tissue may be beyond what is otherwise clinically visible [180]. Poh et al. found LAF extended beyond the clinically visible lesion in 19/20 tumours, in one case extending 25 mm beyond the tumour boundary [180]. Histological and molecular analysis of 36 biopsies in areas with LAF beyond the clinically visible tumour contained either dysplasia/cancer on histology and/or genetic alterations associated with molecular risk in 35 specimens [180]. While these cases were seen in a specialist setting, they provided initial evidence that VELscope™ could differentiate dysplasia from normal oral mucosa.

The diagnostic accuracy of VELscope™ in detecting dysplasia and OSCC has been studied extensively in specialist referral centres [151, 181–185]. It appears that VELscope™ is efficacious in detecting oral epithelial dysplasia and OSCC with reported sensitivities ranging from 30 to 100 % [151, 181–186]. VELscope™ may also uncover additional dysplastic lesions missed under COE [183, 184]. For this reason, VELscope™ appears to be a valuable tool in the follow up of patients with a history of head and neck cancer. However, Mehrotra et al. argue that since not all dysplastic lesions display loss of fluorescence (LAF), its use in routine practice should be discouraged as it may lead to a false sense of security and these lesions may be ignored [151]. Of concern, VELscope™ appears to have a high rate of false positives with reported specificities between 15.3 and 80.8 % [151, 181–186]. This suggests that VELscope™ is a poor differentiator between benign and dysplastic lesions [182, 185]. In particular, inflammatory lesions can also display LAF and act as confounders when using VELscope™ [184]. In general practice, the majority of oral mucosal lesions seen are benign in nature and over-estimation of oral mucosal abnormalities may lead to patient harm through unnecessary biopsies and referrals.

The efficacy of VELscope™ has been extensively assessed at a specialist level on high risk patient groups. One of these studies found that 81 % of areas displaying LAF histologically displayed benign features, although LAF was significantly associated with OED and OSCC compared to normal tissue [181]. Sensitivity and specificity of the device in detecting moderate to severe OED or OSCC were 100 and 80 %, respectively. There were ten false positive (FP) and 42 true negative (TN) findings and the PPV and NPV were 55 and 100 %, respectively. The authors acknowledged that differentiating between LAF and diminished fluorescence was related to the experience of the user, whom in this study were surgeons experienced in using the device. These results are similar to the findings of another single blind study also examining a high risk group using both COE and VELscope™ [183]. Biopsies were conducted on the basis of COE alone, VELscope™ examination alone or on the basis of both techniques. VELscope™ displayed a sensitivity of 92 % and specificity of 77 % whereas COE displayed sensitivity and specificity of 62 and 88 % respectively.

Awan and colleagues achieved different results evaluating VELscope™ on a population selected on the basis of having mucosal lesions requiring biopsy [185].

LAF was noted in 105 lesions, half of which displayed complete LAF, 16 lesions demonstrated retained fluorescence, three displayed increased fluorescence and two had a mixed result. The study noted that 116 out of 126 participants received incisional biopsies but it did not specifically mention which lesions were excluded. VELscope™ displayed a sensitivity and specificity for detection of OED of 84 and 15 %, respectively. The study concluded that the device was unable to discriminate between high and low risk lesions, although it could confirm the presence of leukoplakia and erythroplakia as well as other mucosal lesions.

Mehrotra et al. conducted a cross-sectional study assessing the ViziLite™ and VELscope™ systems on lesions displaying innocuous clinical appearance using Sciubba's classification of Class 1 and Class 2 lesions [151, 187]. ViziLite™ had a sensitivity and specificity of 0 and 76 % whereas VELscope™ had a sensitivity of 50 %, specificity of 39 %, PPV of 6 % and NPV of 90 %. The poor results could be attributed to the author's decision to screen only innocuous lesions as they asserted that screening lesions which displayed high levels of clinical suspicion and required immediate biopsy 'would be meaningless' [151].

The manufacturer's instructions for VELscope™ use include blanching of lesions as part of the examination process. Blanching of lesions allows for evaluation of diascopic fluorescence, which refers to cases in which lesions display LAF but regain complete fluorescence of all areas of the lesion under application of pressure. This phenomenon highlights the absorptive features of haemoglobin associated with oedema and inflammation which can mimic LAF [188]. Despite the importance of this step, only two studies have incorporated blanching of lesions as part of the examination process [182, 184].

Farah and colleagues examined 112 patients who were selected on the basis of having a leukoplakic, erythroplakic or erythroleukoplakic lesion that required evaluation by an oral medicine specialist [184]. Patients with known cases of OSCC or OED were excluded. COE was conducted in accordance with previous publications using incandescent light and was then followed by VELScope™ examination [47, 48]. The results of COE were used to group lesions into categories including homogeneous leukoplakia, non-homogeneous leukoplakia, lesions with lichenoid features and 'other' lesions. The second was a cross-sectional study which featured a similar patient cohort to that reported by Farah with the addition of randomisation of the participants into COE or VELscope™ examination groups [182]. In this study, Rana et al. attempted to reduce the incidence of FP findings by incorporating blanching of lesions into the examination process as well as reviewing lesions of suspected acute inflammatory origin after 2 weeks.

Farah and colleagues calculated an accuracy of 69 % for COE and 55 % for VELscope™ alone. Qualitative data indicated that VELscope™ enhanced visualisation of 35 % of lesions and uncovered five clinically undetected lesions, one of which displayed moderate OED. There were no differences noted regarding border distinctness or visibility between benign lesions and OED. VELscope™ examination resulted in a change of biopsy site in four cases and change in provisional diagnosis in 22 cases. The combined sensitivity of COE in addition to a VELscope™ examination varied significantly with Farah determining 46 % sensitivity while

Rana and colleagues established 100 % sensitivity. Both studies criticised the diagnostic value of diascopic fluorescence with Farah noting that of the 38 lesions which displayed diascopic fluorescence there were ten cases of OED and one OSSC indicating that blanching cannot rule out malignancy.

While the majority of studies have evaluated the diagnostic capabilities and accuracy of VELscope™ without taking into account clinical characteristics, VELscope™ is designed as an adjunctive aid to rather than a replacement for COE. For this reason, Farah and colleagues prospectively evaluated what additional benefits VELscope™ provided when used in conjunction with COE in a specialist environment, in addition to assessing for diascopic fluorescence [184]. Lesions which displayed complete diascopic fluorescence were considered negative for LAF. When combined findings of VELscope™ and COE were considered, the sensitivity was shown to be higher than for either examination alone without a large drop in specificity [184]. This suggests the importance of clinical interpretation when using VELscope™ rather than relying on LAF findings on its own. However, this study was performed by specialists and it is suggested that advanced knowledge of oral mucosal pathology is required to effectively interpret VELscope™ findings and this may not be within the scope of general practitioners [181, 182, 184]. Interpreting VELscope™ findings can be difficult in relation to what constitutes LAF, and diminished autofluorescence is arbitrary and may be vulnerable to inter-operator variability [181]. Farah et al. observed that blanching of lesions is difficult to achieve and partial blanching in particular may complicate interpretation [184], and noted that perhaps the device was potentially more suited in a specialist oral cancer clinic instead of a general dental or medical practice, although further studies were indicated in that context. Success using VELscope™ requires significant operator experience, careful interpretation of findings as well as consideration of the results of COE, as even lesions that display retained autofluorescence or diascopic fluorescence may be dysplastic [182, 184].

The sample populations assessed in the previous studies undertaken in specialist settings could have overstated the positive predictive value of the device [189] and as such evaluation of VELscope™ at a general practitioner level is vital [190, 191].

In a parallel cohort study assessing COE and VELscope™ in a general practice setting, Huff established a 0.83 % prevalence of mucosal abnormalities using COE, none of which were OPMDs and a 1 % prevalence of mucosal abnormalities using VELscope™ of which 83 % were OPMDs [192]. Nine hundred and fifty-nine patients were screened using COE over a period of 1 year followed by examination of 905 patients using VELscope™ the next year. OralCDx brush biopsy or liquid-based brush cytology were performed on the eight lesions detected by COE and 12 lesions detected using VELscope™ as these lesions could not be diagnosed clinically as benign. Two of the eight lesions detected by COE initially displayed mild atypia but were later confirmed as pigmentation and hyperkeratosis. Conversely, all 12 lesions detected using VELscope™ displayed abnormal cells and subsequent histology confirmed the presence of OED in ten cases. Although Huff et al. incorporated a review period to allow the resolution of inflammatory lesions, they found an increased rate of detection of oral epithelial dysplasia using VELscope™ when

compared to COE [192]; however the study was conducted in parallel cohorts, not on the same patient base and the clinical characteristics of lesions discovered with VELscope™ were not discussed. It cannot be determined if the addition of VELscope™ discovered new lesions or helped raise the suspicion of otherwise visible lesions.

Another opportunistic screening study focused on lesions detected by VELscope™ which were not visible during COE [193]. Six hundred and twenty patients were examined using white light and VELscope™ by dental students and qualified dentists. Areas of LAF reported as normal during COE were noted in 69 patients. Assessment of normal variations in tissue characteristics were used to exclude 41 cases; however, blanching was not utilised as a diagnostic criteria and as such FP findings due to inflammation induced LAF were not eliminated. Five patients consented to immediate biopsy and four patients had areas of persistent LAF biopsied at a 2 week review. Five cases of mild to moderate OED were noted as well as two cases of oral lichen planus and two inflammatory lesions.

Only one general population study assessing VELscope™ has calculated a sensitivity and specificity for the device. In this study all lesions detected by VELscope™ were also visible using COE and all lesions displaying LAF upon 2 week review were biopsied [194]. Only 2 of the 32 samples taken were positive for malignancy or dysplasia, and sensitivity and specificity were calculated at 67 and 6 % respectively. McNamara and colleagues found the low specificity of VELscope™ to be a concern arguing the use of VELscope™ in routine screening would lead to a large number of over referrals [194]. In addition, a case of moderate dysplasia of the lip did not display LAF reiterating previous concerns that in general practice, areas of dysplasia may be missed if clinicians become over-reliant on VELscope™. In this protocol, the authors did not consider VELscope™ findings by re-examining areas with LAF clinically and proceeded to biopsy all areas with LAF. It is emphasised that LAF alone has little meaning without clinically re-examining the site to eliminate inflammatory, pigmented or vascular lesions which may contribute to this phenomenon [184]. Clearly the presence of LAF needs to be evaluated in conjunction with the results of COE to identify and eliminate obvious FP findings and prevent patient harm through unnecessary biopsies.

Laronde et al. performed a large-scale multi-centre and multi-operator study to evaluate what benefits fluorescence visualisation with VELscope™ provides above that of a white light examination for routine screening of oral mucosal lesions [195]. Lesions were categorised into high and low risk based on clinical characteristics and fluorescence positive or negative based on VELscope™ findings. Common mucosal changes including amalgam tattoos, fordyces granules, vascularities and pigmentation due to skin colour were excluded. A 3 week review was incorporated to allow for healing of inflammatory lesions and patients were referred to oral medicine clinics as required. Lesion colour and texture were associated with LAF and those deemed high risk were more likely to be present at the 3 week review. The authors found that using fluorescence in addition to a clinical risk assessment provided the best prediction value for lesion persistence when compared to either screening modality on its own. In addition, the strength of models increased when the first

25 % of patients screened were removed which supports previous comments about the importance of clinician experience when interpreting VELscope™ findings. The importance of blanching was not assessed as a part of this study.

Previous studies have focused on the diagnostic accuracy of VELscope™ and its ability to detect dysplasia. However, the device is intended to be used as an adjunct to a clinical examination and not as a stand-alone diagnostic tool [196]. The role of a general practitioner is not necessarily to diagnose dysplasia, but to make appropriate clinical decisions and referrals to a specialist centre where the patient can be diagnosed and managed appropriately. This means referring lesions that appear suspicious, but also knowing when not to refer, to maintain the highest standard of patient care. Therefore in general practice, while assessing the ability to detect dysplasia can be one outcome measure, the ultimate aim of an oral mucosal examination should not be to detect dysplasia, but to detect and appropriately refer oral mucosal abnormalities. In a recent study, Farah and colleagues have shown that one in five people present with an oral mucosal lesion [197]; however it is often difficult to differentiate benign lesions from OPMDs with COE [40].

Given the low specificity associated with the use of VELscope™, there is concern that this would result in a significant increase in the number of specialist referrals which may lead to patient harm through unnecessary stress, and wasted time and financial costs. This was the concern expressed by McNamara et al. who found that a number of benign lesions displayed LAF [194]. This is in agreement with recent findings from a study by Farah and colleagues assessing a decision-making protocol in general dental practice [198], where a number of lesions which were clinically benign displayed LAF. They found that clinical interpretation was extremely important when utilising VELscope™ as relying on LAF findings alone was unreliable. Pigmented lesions, vascular lesions and inflammatory lesions in particular may confound the operator as they also present with LAF [184, 195]. A common finding in this study was the presence of areas of LAF under dentures in clinically normal tissue displaying chronic inflammation [198, 199]. In contrast to the decision-making protocol reported by Bhatia et al., McNamara et al. biopsied all lesions with LAF which did not resolve [194], a finding that does not appear to be warranted [198].

Laronde et al. found that lesion persistence was best predicted using a combination of clinical examination and VELscope™ findings than with either screening modality alone [195], a finding echoed by Bhatia et al. where the combined examination provided a more accurate assessment [198]. Using the decision-making protocol devised by Farah, an additional five patients were identified for referral beyond COE, of which one patient proved to have mild dysplasia. While there was a small drop associated in specificity of referrable lesions from 99.0 to 97.9 %, this was not as significant as previously reported when diagnosis of dysplasia was used as an outcome measure [184, 194]. Compared to the specificity of VELscope™ alone, the combined protocol removed a large portion of over-referrals. The use of the decision-making protocol was also associated with an increase in sensitivity from 44.0 to 73.9 %, while the sensitivity of VELscope™ was 64.0 %. The importance of COE cannot be understated as VELscope™ itself may not detect all significant lesions as it has been found that not all cases of dysplasia display LAF [184].

While previous studies have stated the importance of diascopic fluorescence [184], the significance of blanching has not been assessed in general practice. In the study by Bhatia et al. [198], lesions which partially blanched were associated with the highest rate of referrals. Among reviewed lesions, those with LAF and no blanching tended to show the highest rates of healing although this did not reach significance. This highlights the importance of reviewing patients with LAF and not acting on initial findings. Epithelial dysplasia can be associated with inflammation and changes in underlying vasculature which can contribute to the problem of partial blanching [200]. The higher rate of healing among reviewed lesions with no blanching may be due to the presence of extravasated haemoglobin in acute traumatic events which are a frequent occurrence in the oral environment. Accurate blanching can be difficult to perform and is highly dependent on operator interpretation; however, it forms an essential part of assessment of lesions using autofluorescence. Blanching was more easily performed using the back of a periodontal or sickle probe rather than the back of a mirror particularly with smaller lesions and in difficult to access areas.

Using VELscope™ alone to screen patients in routine general dental practice over-estimates the burden of significant oral mucosal abnormalities and may lead to over-referral. Using the decision-making protocol devised by Farah and colleagues [198], with particular emphasis on careful clinical interpretation and reviewing lesions where appropriate, can result in a decrease in the number of unnecessary referrals which may occur if relying on LAF alone. In addition, VELscope™ may aid in the detection of dysplasia which may not be identified by COE alone.

Overall, VELscope™ can differentiate between normal mucosa and mucosal abnormalities; however, it is not highly specific in detecting OPMDs and as a result gives rise to a high rate of false positives. The sensitivity varies among studies and this could be due to inter-operator variability in what constitutes LAF. It has been reported that there is a large spectrum of fluorescence and more definitive criteria in what constitutes LAF may be required before VELscope™ can gain widespread use [181]. Further, it has been suggested that a significant understanding of mucosal pathology is required to make correct clinical interpretations of VELscope™ findings [184]. This understanding may not be present in a general dental practice, but use of a decision-making protocol may circumvent this problem [198]. If the specificity of the device could be improved, there would be an increased scope for the use of VELscope™ in routine general practice. A new device called Sapphire Plus™ (DenMat Holdings LLC., CA, USA), similar in concept to VELscope™, is the latest adjunctive aid to reach the market; however, currently no literature is available on its efficacy [53].

Identafi®

The Identafi® (DentalEZ, PA, USA) is promoted as an intra-oral, multispectral screening device which incorporates light sources of three different spectra within the one device which are used sequentially to examine oral tissues [201]. In addition to light emitting diode (LED) white light, the device also includes violet (405 nm) and green-amber (545 nm) wavelength lights to induce tissue fluorescence and reflectance spectroscopy, respectively.

The white light is used to illuminate the oral cavity to enhance COE, as white light allows for greater visibility than traditional incandescent light sources [47]. The violet light uses the principles of autofluorescence similar to VELscope™, and previous studies have shown a high sensitivity and specificity using this wavelength to discriminate between normal tissue and dysplasia or invasive carcinoma [167]. The green-amber light is used to delineate abnormal vasculature in the underlying connective tissue. Vascularity has been shown to be a reliable indicator of angiogenesis in oral mucosal lesions [202]. Increased vascularity has been observed to occur at an early stage in the dysplastic process with significant differences found between normal mucosa and mild dysplasia [202]. It is claimed that using the green-amber light can assist the clinician in visualising abnormal vasculature [203]. Currently limited clinical data is available on the use of Identafi® [204].

While a larger trial is ongoing, Lane et al. have released a sample of preliminary cases to illustrate lesion visualisation with Identafi® [203]. Lane et al. observed that using the 405 nm wavelength, areas of loss of fluorescence attributed to breakdown of stromal architecture are often larger than the clinically visible cancer [203]. They also suggested that this is an indication of increased neovascularisation of the stroma [205]. They proposed that these early findings indicate this technology could assist in determining surgical margins for excision of lesions [205]. The green-amber light appears to emphasise keratinisation of tissues and also highlights increased superficial vasculature [205]. A significant increase in microvessel count has been associated with the development of OPMDs [200] and the increased microvessel count noted in mild/moderate OED indicates that angiogenesis may be an early step in tumour progression [200, 202, 206]. Existing evidence indicates that tumour-induced angiogenesis results in altered vascular morphology and is therefore pertinent in determining the status of oral lesions [200, 206]. This supports assessment of angiogenesis in OPMDs to increase the efficacy of early detection and to improve the prognosis of these lesions.

Reflectance spectroscopy has determined that the ideal light required to view underlying vasculature needs to lie within the absorption spectrum of haemoglobin, specifically between 400 and 600 nm [188]. There are significant reductions in the reflectance spectra of OSCCs and OPMDs at 577 and 542 nm which can be attributed to increased light absorption due to increased microvasculature and oxygenated haemoglobin in these lesions. While other imaging modalities evaluating tissue angiogenesis are available, such as Narrow Band Imaging (NBI) [120, 207], Identafi® is the only small hand-held commercially available device utilising tissue reflectance spectroscopy to visualise mucosal vasculature.

Lane et al. found that the green-amber light enhanced the visibility of surface vasculature and the keratotic features of lesions making them larger and more visible [203]. High resolution images of lesions illuminated using green-amber light allowed the examiners to visualise vasculature specific to neoplasia. In addition, taking detailed clinical images using the Identafi® violet and green-amber lights is technique sensitive, and retrospective analyses of such detailed clinical images may not be practical in general practice.

Sweeny et al. evaluated the use of Identafi® in the follow up of 88 patients with a history of head and neck cancer [208]. Both conventional examination and the use of the violet light were associated with a sensitivity of 50 %; however autofluorescence was associated with a decrease in specificity from 98 to 81 %. The use of the green-amber light did not detect any dysplastic lesions providing a sensitivity of 0 %. However, not all areas with loss of autofluorescence were biopsied and reasons for this were not provided. It is possible that these areas had underlying epithelial dysplasia which was not apparent clinically. The authors did not specify what constitutes a positive finding, whether epithelial dysplasia, OSCC or either. Included in the four positive lesions was one at the base of the tongue which can only be accessed via the use of a scope which would not normally be visible under conventional examination or using Identafi®. The study indicated that the use of Identafi® did not provide any assistance above that of COE. This data was based on clinicians with specialist level training and cannot be generalised to include dental practitioners. Furthermore, the white light function of the device was not evaluated and the authors attributed the low sensitivity noted to post radiation induced changes, such as fibrosis and pigmentation.

Preceding publications place little emphasis on the importance of the white light function of the Identafi® and the results cannot be generalised as the screening clinicians in those studies had specialist level training [203, 208]. Identafi® is the most recent development in commercially available oral cancer screening devices, incorporating assessment of tissue autofluorescence and tissue reflectance spectroscopy. This device has not been assessed clinically on a general population or with uniform histological correlation and as such the relevance of the device to general or specialist dental practice is yet to be determined. A new device termed DOE SE™ (DentLight Inc., TX, USA) appears to be similar to Identafi® and also uses violet light for autofluorescence however currently no clinical trials are available on the device [54].

Farah and colleagues [209] have assessed the utility of all three lights of the Identafi® for the assessment of oral mucosal lesions in an oral medicine specialist setting and found that Identafi® white light provided visualisation of the oral cavity equivalent to that produced by an overhead LED white light source supplemented by 2.5x magnification. LAF using Identafi® violet light was highly associated with clinical diagnosis ($p = 0.002$) with lesions displaying lichenoid features more likely to reveal moderate to significant LAF using the violet light compared to RF, while homogeneous lesions were more likely to display RF [209].

Diascopic fluorescence was highly associated with clinical diagnosis ($p = 0.0001$), with non-homogeneous lesions more likely to display incomplete blanching while lesions with lichenoid features more commonly displayed diascopic fluorescence. LAF appears to display high efficacy for detection and monitoring of inflammatory pathology, such as oral lichen planus, whereas use of the device to examine non-homogeneous lesions suspicious for underlying OED is technique sensitive and requires a high level of clinical skill and interpretation. The Identafi® violet light displayed a sensitivity of 12.5 % and specificity of 85.4 % for detection of dysplasia on histopathology; with a negative predictive value of 59.4 % and a positive

predictive value of 36.4 % indicating that autofluorescence alone cannot accurately and consistently differentiate between OPMDs and benign lesions. While clinically visible vasculature was noted in 40.9 % of lesions in the study by Farah and colleagues [209], visibility scores and mean lesion size were lowest when using the green-amber light. The discrepancy in these results and that of Lane et al. could be attributed to study design, as Lane and colleagues retrospectively analysed high resolution images of lesions illuminated using green-amber light. Sweeny and colleagues found that the green-amber light displayed a sensitivity and specificity of 0 % and 86 % respectively [208] but did not specify if underlying vasculature was visible or if the green-amber light enhanced the visibility of keratotic features of lesions, as noted by Lane et al. [203]. In the study by Farah and colleagues, tissue vasculature could not be visualised using the green-amber light in 59.1 % of lesions. This could be attributed to the high proportion of lesions displaying keratotic features (68 %), as lesion features were highly associated with visibility of diffuse vasculature ($p=0.0001$). Lesions displaying keratotic features were significantly less likely to display clinically visible vasculature using the green-amber light.

Lesions of a homogeneous clinical appearance frequently have keratotic features and it is not surprising that these lesions were also less likely to display clinically visible vasculature ($p=0.0002$). The green-amber light of Identafi® is intended for use in a differential manner following examination using the white and violet lights [201]. For this reason, the relationship between lesion characteristics using both lights was also examined. LAF was associated with moderately or significantly diffuse vasculature while lesions displaying RF were unlikely to display clinically visible vasculature using the green-amber light ($p=0.0001$). A greater proportion of lesions with visible vasculature also displayed diascopic fluorescence but this did not reach statistical significance. Therefore lesions which are detected during COE but display RF using the violet light are also unlikely to be detected using the green-amber light.

Based on their assessment of OPMD, Farah and colleagues [209] note that Identafi® shows potential for use as an adjunct to COE for detection and visualisation of oral mucosal lesions, and that its white light produces superior visualisation of the oral cavity compared to incandescent or extra-oral light sources. The violet light displays a high level of clinical utility for evaluating inflammatory pathology but poor sensitivity for detection of OED. The green-amber light provides additional clinical information in relation to underlying vasculature and inflammation of lesions. The strength of Identafi® over other visual diagnostic aids lies in the combination of its multispectral light sources, each of which appears to add useful clinical information for better visualisation of oral mucosal lesions when used sequentially.

9.5 Narrow Band Imaging

Narrow Band Imaging (NBI, Olympus Medical Systems Corporation, Tokyo, Japan) is an endoscopic technique that provides real-time on-demand optical image enhancement of the mucosal and submucosal vascular morphology and mucosal surface texture [210] [207]. The technology utilises the concept that the wavelength

of light determines the depth of penetration [210, 211]. Two 30 nm wide bands of blue and green light are filtered from white light and are emitted simultaneously in NBI mode. The capillary bed and intrapapillary capillary loop (IPCL) pattern appears brown due to the blue light centred at 415 nm, as this wavelength corresponds to the peak absorption spectrum of haemoglobin. Thicker blood vessels in the deeper mucosa and submucosa appear cyan due to the green light centred at 540 nm [210–213]. As angiogenesis is associated with potentially malignant and malignant lesions, the ability for NBI to enhance the microvascular architecture enables clinicians to delineate diseased tissue from normal mucosa [16].

In NBI mode, two optical filters placed in front of WL select two narrow bands of light in the blue and green spectrum. Blue light between 400 and 430 nm (centred at 415 nm) corresponds to the peak absorption spectrum of haemoglobin and can therefore highlight the capillary bed and intrapapillary capillary loop (IPCL) pattern in the superficial mucosa by making them appear brown. Thicker blood vessels in the deeper mucosa and submucosa are enhanced by green light between 525 and 555 nm (centred at 540 nm) and appear cyan [210–213]. A charge coupled device (CCD) at the tip of the endoscope captures the reflected light, which is then reconstructed to produce a coloured NBI image that is displayed on a monitor. Switching between WL mode and NBI mode simply involves pressing a button on the videoendoscope, video camera or monitor console [13]. Magnifying endoscopy, which can enhance morphological and colour changes in the mucosa and allow for clearer visualisation of microvascular structures, is also possible with the two commercially available NBI systems [210, 214]. The red-green-blue sequential NBI endoscopes (Evis Lucera 260 Spectrum) can optically magnify images up to 80 times and is considered to give clearer images, whereas the colour CCD endoscopes (Evis Exera II and Evis Exera III) are coupled with digital zoom at 1.2 and 1.5 times magnification. Both are capable of maintaining excellent resolution even when the endoscope tip is as close as 2 mm from the mucosal surface due to their physical zoom property [214].

As angiogenesis occurs early in the carcinogenesis continuum, the distinct microvasculature architecture associated with potentially malignant and malignant lesions can be used to differentiate these lesions from normal mucosa [16, 19, 215]. Areas of neoplasia are typically characterised by well-demarcated brownish areas with scattered spots, whereas inflammatory lesions have ill-demarcated borders [213, 216]. However, NBI has been designed to enhance microvascular morphology and can therefore be used to detect vascular changes such as the degree of dilation, meandering, tortuosity and calibre of IPCLs [16, 215]. Typically, a separate IPCL classification for oral mucosa is used for oral lesions [216]. This classification is a simplified version of Inoue's IPCL classification for oesophageal mucosa [215]. Normal mucosa has IPCL type I and is characterised by regular brown dots when loops are perpendicular to the surface of the mucosa, or waved lines when parallel. Non-neoplastic lesions are either type II, which has a dilated and crossing IPCL pattern, or type III, which demonstrates an elongated and meandering IPCL pattern. Neoplastic lesions have type IV, which is characterised by large vessels, IPCL pattern destruction and the presence of angiogenesis. For all these classifications, the

most advanced IPCL pattern determines the type of lesion when more than one pattern is present [215–217].

Although NBI is commonly used in the gastrointestinal, aerodigestive and urinary tracts, the use of this technology in the oral cavity to screen for oral potentially malignant lesions (OPMLs) and OSCC has only been a fairly recent development. Consequently, the literature regarding the use of NBI as a visualisation adjunct for screening potentially malignant and malignant lesions in the oral cavity and oropharynx is still limited [207]. Nonetheless, NBI has demonstrated high sensitivity and specificity for aiding the detection of dysplasia and neoplasia elsewhere in the head and neck [218].

Early detection of malignant and potentially malignant mucosal lesions in the head and neck is critical for improving patient prognosis as treatment is less invasive [219]. Although the use of WL in the oral cavity has relatively high sensitivity and specificity [116], early lesions may be present in clinically normal mucosa and can therefore be easily missed with WL examination [41]. Many visualisation adjuncts on the market aim to improve the detection rates of dysplasia and cancer; however, most have issues with differentiating benign lesions from dysplasia and neoplasia [120].

NBI has a higher diagnostic accuracy than WL for aiding the detection of OPML, OSCC and/or OPSCC with consistently high values across the board for NBI [220, 221], which suggests that the overall false positive and false negative rates tend to be low with the use of NBI. However, it is still unclear whether or not NBI has better sensitivity, specificity, PPV and NPV than WL based on these two studies alone [220, 221]. In one study, NBI alone and NBI with HDTV had higher sensitivity and NPV but lower specificity and PPV than WL with HDTV [220]. This was the reverse with the other study, with higher specificity and PPV but lower sensitivity and NPV reported for NBI in comparison to WL [221]. Combining WL and NBI classifications did not necessarily result in improved diagnostic accuracy across the board, as Yang et al. reported that while sensitivity and NPV improved, specificity, PPV and accuracy dropped to almost the same, if not the same, as the WL values [221]. Instead, use of NBI classification alone was significantly better than using WL or combined WL and NBI classifications for aiding the detection of HGD or worse [221].

Several other papers have also reported the effectiveness of NBI over WL for aiding the detection of dysplasia and cancer [218, 222]. The only published meta-analysis of studies assessing the diagnostic accuracy of NBI in the oral cavity and/or oropharynx calculated 92% sensitivity, 95% specificity, 25.11 positive likelihood ratio (PLR) and 0.09 negative likelihood ratio (NLR) [218]. In comparison, WL had 50% sensitivity, 100% specificity, 21.10 PLR and 0.52 NLR [218]. Based on the information provided, one retrospective and two prospective studies were included for this meta-analysis [219, 223], and of these three studies, only one met the inclusion criteria for the current review [219, 224]. The validity of this meta-analysis must be questioned as one of the included prospective papers is an abstract that appears to be an earlier report of the other included prospective paper [219, 224]. Nonetheless, other studies report similar values of efficacy, with a recent study

investigating the use of NBI for aiding the detection of OSCC in chronic oral ulcers persisting for longer than 3 weeks noting 93.75 % sensitivity, 91.49 % specificity, 78.95 % PPV, 97.73 % NPV and 92.06 % accuracy [222]. It is clear that NBI has very high accuracy for detecting potentially malignant and malignant mucosal lesions in the oral cavity and oropharynx. Other studies that have used NBI in the head and neck and have found oral and oropharyngeal lesions have also reported similar values of efficacy for NBI [225, 226].

A prospective study by Nguyen et al. used WL, autofluorescence and NBI to examine the oral cavity, hypopharynx, larynx and bronchus of 73 patients with HNSCC, SCC of unknown primary origin or previously treated HNSCC patients who were thought to have recurrent disease [226]. Of these, 25 patients had a primary tumour site in the oral cavity. The authors reported 96 % sensitivity and 79 % specificity for moderate dysplasia or worse by NBI, whereas the sensitivity and specificity for autofluorescence was 96 and 26 % respectively, and for WL it was 37.5 and 95 % respectively. Detection of significant dysplasia or worse was therefore significantly better with NBI than with WL, and both NBI and WL had less false negative findings than autofluorescence. The use of autofluorescence and NBI significantly affected the immediate management of three oral cases – namely, they assisted with mapping the surgical margins. This finding is supported by not only Piazza et al. but also other studies that have reported the value of NBI in determining the resection margins of OPMLs and OSCCs [216, 227, 228]. By using NBI prior to excision, the true extent of a lesion can be determined such that complete resection is possible [227]. NBI also influenced the long-term follow-up of one oral lesion with confirmed moderate dysplasia that had persistent NBI changes [226].

Knowledge of the IPCL pattern can influence the subsequent course of care because the likelihood of more serious pathology being present increases with each stepwise increase in the IPCL pattern type [216, 229]. As shown in the study conducted by Yang et al., the IPCL pattern shown by NBI correlates with the pathological severity of oral leukoplakia better than using clinical morphological features of leukoplakia [221]. In non-neoplastic and non-inflammatory lesions, there are no irregularities in IPCL; however, once inflammation occurs, IPCLs will proliferate, elongate and dilate slightly. Furthermore, with dysplastic lesions, the IPCLs not only increase in density, dilation and calibre, but also extend upwards, proliferate and branch irregularly in accordance with the degree of dysplasia. The lesion becomes thicker due to the increased microvascular density (MVD) and will eventually result in subepithelial invasion and destruction of IPCLs if left untreated. By this stage, the lesion becomes an invasive SCC. As there is a significant correlation between the thickness of intraepithelial lesions, MVD and subepithelial invasion, the detection of malignant and potentially malignant mucosal lesions at an early stage is very important [16].

The idea of early detection is supported by a study involving 154 patients with newly diagnosed leukoplakia. In this study, the authors reported 16.67, 92.31 and 100.00 % frequencies of dysplasia in lesions with IPCL types I, II and III, respectively. All lesions with IPCL type IV were histopathologically confirmed as SCC, and this suggests that IPCL type IV could be pathognomonic of OSCC [230].

A different study which used three microvascular patterns very similar to the types II, III and IV IPCL patterns outlined in Takano's IPCL classification for oral mucosa confirmed these findings [216, 231]. In this study, the IPCL types II and III equivalents were associated with premalignant and carcinomatous lesions, whereas the IPCL type IV equivalent was only present in OSCC [231]. The sensitivity, specificity, PPV, NPV, accuracy and OR for detecting HGD, CIS and invasive carcinoma using IPCL types III and IV as the criteria for differentiating neoplastic mucosa from normal mucosa were generally very high at 84.62, 94.56, 74.32, 97.06, 93.0 and 95.53 (95% CI: 42.19–216.29) respectively [230]. Consequently, lesions with IPCL types II, III or IV under NBI illumination should be biopsied [229].

A retrospective study also found a significant association between types III and IV IPCL patterns and the presence of OSCC in non-healing ulcers, with the sensitivity, specificity, PPV, NPV and accuracy for using types III and IV IPCL patterns as the diagnostic criteria at 93.75, 91.49, 78.95, 97.73 and 92.06%, respectively [222]. While a chronic ulcer in itself is not considered an OPML by the World Health Organization [25], it can be a sign of malignancy [222]. This study further demonstrates the diagnostic utility of intraepithelial microvascular morphology for determining the presence of neoplasia.

While Yang et al. utilised IPCL patterns to identify the presence of disease under NBI [221], Piazza et al. used a more basic criteria and instead looked for the presence of a 'well-demarcated brownish area with thick dark spots and/or winding vessels' [220]. The scattered brown dots represent the superficial microvessels, whereas the intervascular brownish epithelium may be due to increased intraepithelial cell density or the inherent changes in intraepithelial cells that occurs during malignant transformation [232]. Use of this criterion for detecting HGD, CIS or SCC is less effective than by using the IPCL patterns [223]. Furthermore, recent research has shown that the prevalence of brownish spots is not consistent in all areas of the head and neck [233]. Variations in the epithelium such as the degree of keratinisation and thickness, and the presence of lymphoid tissue can affect visualisation of the subepithelial microvasculature architecture and IPCLs [233].

For NBI to be effective, light must be able to penetrate the epithelium. The oral cavity has several different types of epithelium depending on the location, and Lin et al. investigated the effect this had on the appearance of brownish spots [233]. In their study, 125 patients with early or occult mucosal head and neck cancer were examined and then split into two groups according to the presence or absence of brownish spots. There was a significantly higher prevalence of brownish spots (OR = 76.45) in areas lined with non-keratinised thin stratified squamous epithelium such as the floor of mouth, ventral tongue and soft palate, than in areas lined with thicker (i.e., greater than 500 μm) or keratinised epithelium [233]. In contrast, another study reported that visualisation of the microvasculature is not affected by the degree of keratinisation in normal mucosa, unless there is hyperkeratosis associated with leukoplakia [230].

Several studies have reported impaired visualisation of the microvascular network in the presence of leukoplakia [216, 225, 230]; however, Yang et al. reported that it is still possible to observe the underlying vasculature under thin homogenous

leukoplakia [229]. In Yang's study, only type I IPCL pattern was seen in thin homogenous leukoplakia, and the majority were histopathologically diagnosed with squamous hyperplasia. Conversely, visibility of the microvasculature was vague, blurry or completely obstructed where there was thick homogenous leukoplakia. Instead, examination of the mucosa surrounding the lesion was necessary to determine the most likely IPCL pattern for the lesion. Interestingly, 75.20 % of thick homogenous leukoplakia were surrounded by IPCL type I, and of these, 27.66 % had dysplasia. Therefore, the IPCL pattern of the surrounding tissue may not be indicative of the actual pattern under the hyperkeratosis. The fact that types II and III IPCL patterns could be observed around thick homogenous leukoplakia but not under thin homogenous leukoplakia, however, suggests that the amount of hyperkeratinisation may correlate to the degree of dysplasia [229]. Suspicion should also be increased if non-homogenous leukoplakia is present, as these lesions are more likely to have high grade dysplasia, CIS or invasive carcinoma than homogenous leukoplakia [223, 230]. Visualisation of the underlying microvasculature may also be impaired in ulcerated lesions due to the presence of fibrin slough or pseudomembrane [222].

Although there are still physiological and anatomical issues that affect the visualisation of the superficial microvasculature [230, 233], NBI is safe, fast and well-tolerated [222]. The main limiting factor is the steep learning curve associated within the first 6 months of using the NBI system; however, once the clinician has passed this phase, using and interpreting results becomes easier [219, 221]. All studies have been conducted by specialists in a specialist setting, and thus the results cannot be generalised to general practitioners or the general population. It is important to note that the use of NBI is not intended for general dental or medical practitioners due to the cost involved in NBI system setup and the level of training required for effective use.

Existing data suggests that NBI has great potential to not only accurately aid the detection and real-time assessment of new and existing OPML, OSCC and OPSCC, but also influence their treatment. Although data regarding the efficacy of NBI for aiding the detection of OPML, OSCC and OPSCC is still limited, with the majority of published papers being case reports [227, 228], retrospective studies [221–223, 229–231] and a few prospective studies [216, 219, 225], there is building evidence to suggest its beneficial use over WL alone for detecting and monitoring mucosal lesions in the oral cavity and oropharynx [207].

Although the prevalence of malignancy may be less than 1 % in some countries, the proportion of patients presenting with an oral mucosal lesion is usually greater [234]. However, OPMDs tend to have more subtle changes in the epithelium than OSCC and can therefore be difficult to detect with standard WL examination [15, 19].

The effectiveness of NBI is affected by the degree in which light can penetrate the epithelium. Farah and colleagues have investigated the use of NBI for assessment of OPMD [234] and found that the IPCL pattern was not visible for 45 out of the 217 lesions with only keratosis. Lesions without keratosis or erosion were significantly more likely to have completely visible IPCLs, and the presence of keratosis reduced IPCL visibility. This is consistent with other studies that have reported

impaired visualisation in areas affected by hyperkeratosis associated with leukoplakia [216, 225, 230]. In another paper by Yang et al., the IPCL pattern was visible in areas with thin homogeneous leukoplakia, but became blurry, vague or completely obstructed in areas with thick homogeneous leukoplakia. With the latter situation, the authors suggested examining the area surrounding the lesion to determine the possible IPCL pattern, although they noted that this did not necessarily represent the actual pattern of the lesion itself [229]. Therefore, rather than assess the area surrounding the lesion, Farah and colleagues included an additional category, type 0, for lesions where the IPCL pattern was completely obscured within the confines of the lesion borders [234].

Although several studies have used IPCL patterns to differentiate dysplasia and neoplasia from normal mucosa with success, Farah and colleagues found no statistical significance between IPCL pattern and the diagnosis of an OPMD or worse [234]. These findings suggest that the vasculature of OPMDs does not appear distinctly pathological as it does for OSCCs. With the variety of conditions included under the category of OPMD as defined by WHO, the microvasculature and surface mucosal changes for the different types of lesions may not be as markedly different from normal tissue as OSCCs.

NBI enhanced the visualisation of lesions detected by COE and WL, aided the detection of lesions that were undetected by COE and WL, and correctly changed the clinical provisional diagnosis of one lesion. In contrast, WL enhanced the overall visibility of lesions compared to COE and aided the detection of lesions that were missed by COE, but did not change the clinical provisional diagnosis of any lesion [234]. These findings are consistent with other studies that have reported improved visualisation, detection rates and management of oral lesions with NBI [219, 225, 226]. However, the advantage that WL and NBI have over COE is the fact that both have 1.5x digital magnification in addition to a physical zoom property that allows the endoscope tip to be as close as 2 mm away from the mucosal surface [214]. Consequently, it is possible to visualise lesions and the detailed features of lesions that may otherwise be missed from an extra-oral point of view.

In the study by Farah and colleagues [234], the provisional diagnoses of three lesions were incorrectly changed to an OPMD as the examiner interpreted their IPCL pattern as type III. This highlights the subjective difficulty in interpretation of the nature of IPCLs. It is well known that there is a degree of training required to properly interpret IPCL patterns, and a steep learning curve associated with using NBI and interpreting mucosal changes and underlying microvasculature [219].

The high sensitivity, PPV, NPV and accuracy of NBI compared to COE or WL confirms that NBI has low rates of false negatives. NBI had lower specificity than both COE and WL, suggesting that there is a higher rate of false negatives with NBI. However, the gold standard comparison was either COE or WL, both of which detected fewer lesions than NBI. Although this study had a clinical focus in order to compare NBI with existing gold standards – namely, COE for non-endoscopic visual assessment and WL with magnification when using the endoscope, the concern with using another light as the gold standard comparison is that new lesions were technically considered false positives if it was not detected with the other light modality.

For the subset of lesions that were biopsied however, the sensitivity, specificity, PPV, NPV and accuracy was lower for NBI when histopathology was used as the gold standard [234]. This is attributable to the small sample size, the majority of biopsies taken without input from NBI, lack of multi-site biopsies and examination by a single pathologist. Nonetheless, the poorer efficacy values are consistent with the fact that there appears to be no clear association between IPCL pattern type and histopathology at this stage for low-risk OPMDs. Given that many existing oral visualisation adjuncts tend to be poor at differentiating benign and inflammatory lesions from OPMDs and neoplastic lesions [48, 142, 151, 181, 184], further research is required to determine if there are any differences in the microvascular appearance of these lesions by NBI.

Use of NBI in the oral cavity does present with some challenges [234]. Learning how to position the endoscope to provide a clear image on the monitor resulted in poor ergonomics at times, and this is further exacerbated if the monitor was poorly positioned in relation to the operator. Despite the fact that the NBI system is mounted on a freely moveable frame, the device is large and not overly portable. A clinical assistant is recommended for aiding the retraction of oral tissues to improve the clinician's ability to accurately place the endoscope in an ergonomic position, particularly when observing intra-oral areas with overlapping or mobile tissues [234].

Although the microvascular IPCL pattern of OPMDs may not distinctly correlate with pathological diagnoses of OPMDs, NBI demonstrates great utility as a visualisation adjunct for detecting and visualising OPMDs as it has high diagnostic accuracy and can aid the detection of lesions that may not be identified by COE or WL examination alone [234]. Enhanced detection and monitoring of OPMDs with NBI technology has the potential to improve patient outcomes; an area of research that requires longitudinal studies.

9.5.1 Other Optical Imaging Techniques

It has now been established that molecular profiling of tissue changes enable clinicians to 'visualise' more of the disease. While macroscopic changes may be detected under white light examination and tissue/cell level changes through histopathology, molecular dysregulation may be identified using special imaging techniques. While most current methods assess tissue in the plane parallel to the lesion, methods aiding assessment in the vertical cross-section (plane perpendicular to the mucosal surface) are required to detect lesions below the mucosal surface and evaluate submucosal tumour invasion [235].

All optical imaging techniques detect and analyse backscattered photons from mucosa [235]. Visible light (400–700 nm) is used for conventional white light inspection, however shorter wavelengths in ultraviolet (UV) and longer wavelengths in the near-infrared (NIR) regions of the light spectrum can also be used for imaging. UV and blue light are absorbed by biomolecules to produce fluorescence [235]. In order to detect targeted tumour cells, the tumour-specific signal must be significantly discriminated from the non-specific background signals, thus optimising the

signal-to-background ratio (SBR) [236]. The visible light spectrum has relatively short penetration depths useful for imaging (<100 μm) as it is mostly absorbed by haemoglobin and is significantly associated with a high level of nonspecific surrounding signals, resulting in a low SBR [235, 236]. NIR is less susceptible to tissue scattering and haemoglobin absorption, yielding penetration depths >1000 μm through the mucosa and a high SBR, with an optical imaging window of about 650–900 nm in which the absorption coefficient is at a minimum [235, 236].

Optical imaging techniques using Optical Fluorescence Imaging (OFI) and Narrow Band Imaging (NBI) reflect tissue changes at the microscopic and molecular levels. Optical Coherence Tomography (OCT) and Angle-Resolved Low-Coherence Interferometry (a/LCI) non-invasively provide information in the vertical and axial planes. Raman spectroscopy is a point detection technique based on the inelastic scattering of light, also enabling molecular histopathological examination. Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) are used to detect carcinoma and metastasis (hence staging), and assess treatment response, providing anatomical and physiological information. Positron emission tomography (PET) is a true form of molecular imaging, allowing for drug delivery and molecular surgical guidance. Hybrid imaging methods, PET/CT and PET/MRI, offer the best of both these imaging approaches. All these methods, collectively termed 'optical biopsy', are non-destructive *in situ* assays of mucosal histopathologic states using the spectral and spatial properties of scattered light to measure cellular and/or tissue morphology, providing an instantaneous diagnosis [235, 237].

9.5.1.1 Optical Coherence Tomography

Optical Coherence Tomography (OCT) is based on the principle of low-coherence interferometry [235]. It provides high resolution (~1–20 μm) cross-sectional images of tissue *in situ*, higher than conventional ultrasound, MRI or CT, and comparable to conventional histology but being non-destructive, it aids real-time surgical diagnostics and an 'optical biopsy' of the tissue [238]. Initial success with this modality was with retinal pathology [239] and bronchopulmonary diseases [240]. More recently, it has been deemed useful in diagnosing diseases of the oropharynx/larynx and other oral tissues [238, 241, 242].

OCT is similar to ultrasound B-mode imaging except that OCT uses light instead of acoustic waves, measuring the echo time delay and intensity of backscattered light [243]. The system uses NIR light, split into reference and sample beams, and plots the back-reflected light from structures within the tissue against depth (up to 2–3 mm) [239, 243, 244]. Since the velocity of light is extremely high, optical echoes cannot be measured directly by electronic detection, but instead uses low-coherence interferometry – the back scattered light waves interfere with the reference beam and this interference pattern is used to measure the light echoes versus the depth profile of the tissue *in vivo* [244]. OCT also uses fibre optic technology, allowing for low-profile imaging to be performed through small optical fibres attached directly to a scalpel, tissue probe, endoscope or microscope [244]. The device is compact and portable [244].

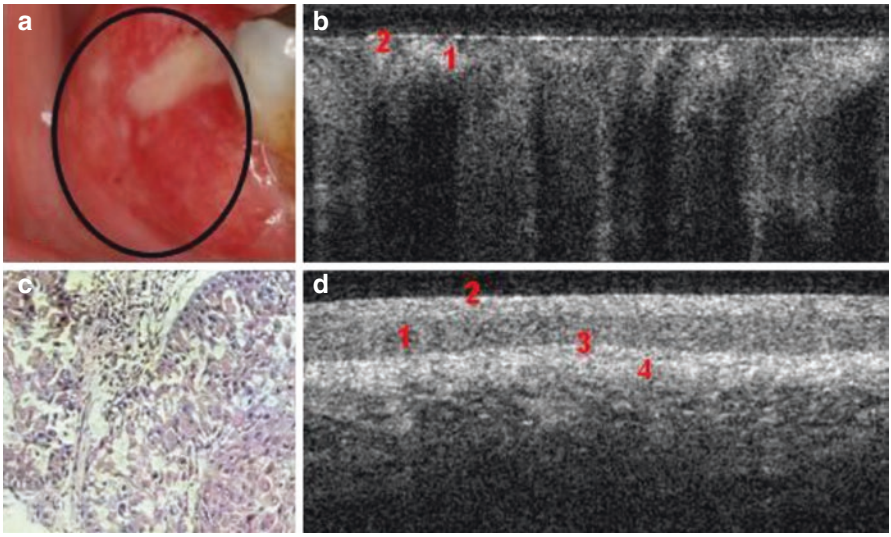

Fig. 9.5 (**a**) Clinical photograph (the *black circle* denotes the clinical lesion), (**b**) *In vivo* OCT image and (**c**) H&E (10x) of buccal mucosa with squamous cell carcinoma. (**d**) In vivo OCT image of normal buccal mucosa. *Key*: *1*-stratified squamous epithelium, *2*-keratinized epithelial surface layer, *3*-basement membrane, *4*-submucosa. From: In-Vivo Diagnosis of Oral Dysplasia and Malignancy Using Optical Coherence Tomography: Preliminary Studies in 50 Patients. Wilder-Smith et al. 2009 (Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc)

In healthy mucosa, the basement membrane can be easily identified at the junction of the bright lamina propria and the darker epithelium, which is lost in the presence of invasive cancer [245]. Figure 9.5 provides an example of OCT imaging of oral squamous cell carcinoma and normal buccal mucosa. However, one study had inconsistent results, showing a deceptive change in the histological layers when compared to conventional biopsy of oral lesions (various anatomical sites) [238]. The authors also noted that OCT image analysis is unique, requiring special training and associated with a wide range of variability when interpreting its parameters (mainly epithelium thickness and status of basement membrane) [238]. The authors previously aimed to generate a bank of normative and pathological OCT data from oral tissues to identify cellular structures of normal and pathological processes, thus creating a diagnostic algorithm [246]. While OCT is useful for clinical detection of OSCC and OPML [247], it also has potential in evaluating surgical margins for minimal residual disease (MRD) in HNSCC just as it has been proven useful in cancers of other tissues such as breast [248, 249], skin [250, 251], vulva [254] and prostate [255].

9.5.1.2 Angle-Resolved Low-Coherence Interferometry

Angle-resolved low-coherence interferometry (a/LCI) is a light scattering technique which isolates the angle scattering distribution from cellular nuclei at various tissue depths [237]. In doing so, it is able to provide biomarkers based on morphology that

are highly correlated with the presence of dysplasia [237]. It measures the angular intensity distribution of light scattered by a tissue sample, quantifying subcellular morphology as a function of depth in the tissue [237]. For each depth layer, signatures from cell nuclei are extracted by collecting and processing the angular scattering signal using a Mie theory-based light-scattering model to produce measurements of average nuclear diameter with submicron-level accuracy [237]. Studies that have investigated the use of a/LCI have confirmed that neoplastic tissue transformation is accompanied by an increase in the average cell nuclei size [237, 256–256], thus detecting potentially malignant lesions as well as malignant lesions. The diameter of a non-dysplastic epithelial cell nucleus is typically 5–10 μm, while dysplastic nuclei can be as large as 20 μm across [257]. When this is optimised to 11.84 μm for the classification of tissue health, a/LCI yields a sensitivity of 100 %, specificity of 84 %, overall accuracy of 86 %, positive predictive value of 34 % and negative predictive value of 100 % in oesophageal epithelium *in vivo* [237, 255]. This technique has been studied in animal models, *ex vivo* human studies, and more recently in *in vivo* studies, predominantly associated with cases of Barrett's Oesophagus (which is associated with an increased risk of oesophageal adenocarcinoma) and oesophageal epithelium [237]. The system is portable and the probe can be used through the accessory channel of a standard endoscope, thus providing surgical guidance [237].

9.5.1.3 Raman Spectroscopy

Raman spectroscopy is a non-invasive technique that can analyse the molecular composition of a tissue, enabling surgeons to identify, examine and determine the quality of the tumour's molecular margins [245]. It is based on the phenomenon that intramolecular bonds cause light to scatter in a manner that is both measurable and predictable, albeit for a very short time constituting <1 part per million of the total reflected light [245]. Point detection techniques can be used to collect molecular information during endoscopy with optical fibre probes, and they have the potential to be extended to imaging [235]. Raman spectroscopy produces inelastic light scattering (returning photons have longer wavelength than the incident photons) and diffuses NIR photons (photons that return after several scattering events and are useful for measuring fine pathological structures) which aid molecular histopathologic examination [235]. It is performed by illuminating tissue with NIR photons that are absorbed by the vibrational/rotational nodes of molecular bonds associated with chemical functional groups specific to mucosal proteins, lipids and nucleic acids [235, 258, 259]. Some of these photons are then inelastically scattered forming detailed spectral patterns that can be reduced to the principal components using multivariate statistics. However, the Raman effect is much weaker than fluorescence and can be easily obscured by fluorescence from the tissue or optical fibre itself [236].

Shim et al. demonstrated the use of CCD detector in collecting Raman spectra *in vivo* in the gastrointestinal tract [260]. Molckovsky and colleagues showed that Raman spectroscopy could be used to distinguish between adenomatous and hyperplastic polyps in the colon, with 100 % sensitivity, 89 % specificity and 95 % accuracy when used *in vivo* [261]. Haka et al. used Raman spectroscopy to examine breast tissue *in vivo* and reported perfect sensitivity and specificity when using

their diagnostic algorithm [262]. They highlighted the feasibility of using it for real-time intraoperative margin assessment during partial mastectomy surgery, which could be similarly used for intraoperative margin assessment in HNSCC cases. Stone et al. examined biopsy specimens of laryngeal mucosa using Raman spectroscopy and conventional histopathological analysis, and reported 92 % sensitivity and 90 % specificity for Raman spectra generated over 30 s in the diagnosis of invasive cancer (compared to reference spectra generated from histopathologically normal mucosa) [263]. In membranous vocal cord specimens, Lau et al. reported 69 % sensitivity and 94 % specificity for invasive carcinoma using Raman spectra recorded over 5 s [264].

Spatially offset Raman spectroscopy (SORS) has been shown to be an effective tool in recovering Raman spectra from up to several millimetres beneath the surface of turbid media [265]. Keller et al. found that, using source-detector separations of up to 3.75 mm, SORS can detect sub-millimetre-thick tumours under a 1 mm normal layer, and tumours at least 1 mm thick can be detected under a 2 mm normal layer using the Monte Carlo simulation model of breast tumour margin analysis [265]. Other recent developments within Raman spectroscopy include surface enhanced Raman spectroscopy (SERS), coherent anti-Stokes Raman spectroscopy (CARS) and stimulated Raman scatters (SRS) [266, 267], which could all have applications in HNSCC margin analysis. Visualising molecular information using Raman spectroscopy has also been shown to aid in identifying patients with prostate cancer who are at risk of cancer progression from those with no evidence of disease [268].

Raman spectroscopy provides an objective analysis of the tissue's molecular structure compared to the *ex vivo* histopathological analysis and grading based on tissue morphology. It may provide a more clinically relevant measure of the tumour margin on which to guide surgical excision. It has been possible to stage and grade malignancies from a spectral measurement on the surface of bladder tissue using Raman spectroscopy [269]. Representative reference spectra need to be developed by analysing a large cohort of histologically diagnosed mucosal lesions, against which spectra captured *in vivo* can be compared and leading to algorithms that can quickly produce a diagnosis [245].

While OFI and NBI can detect tissue and molecular changes in a localised region, imaging modalities such as Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) provide anatomical information, including nodal involvement and metastasis which influence staging and treatment protocol employed. Ultimately, multimodal imaging can provide additional diagnostic information than white light illumination or a single imaging modality alone [163, 270].

Both CT and MRI involve 3D sectional imaging and have extremely high diagnostic value [271]. CT scans require ionising radiation (with shorter scan times) while MRI does not but has a longer scan time [271]. CT is currently the most commonly used modality for head and neck imaging, and can improve delineation of soft tissue pathologies with intravenously administered contrast media [271]; however MRI provides the most detailed view of soft tissues and is routinely used to visualise such tumours [271].

9.5.2 Optical Molecular Imaging Using Exogenous Molecular Probes

Molecular-specific imaging modalities have the potential to be indispensable in every aspect of cancer care, from early detection to staging, drug delivery, molecular surgical guidance and treatment response [272–274]. Oncological molecular imaging is defined as the non-invasive imaging of distinctive cellular and subcellular events in malignant cells [272, 275]. Molecular imaging probes target the production of genetically determined biomolecules by cancer cells by displaying these directly in or on individual malignant cells, in the extracellular matrix, or cells in the vicinity such as T cells, macrophages, dendritic cells, fibroblasts or endothelial cells [276–278]. For example, probes paired with positron emitters and novel target-specific anticancer drugs could be quantitatively imaged by PET, providing information on tumour biology, guiding drug development and furthering personalised medicine [52, 279]. Diseased tissue may also be detected through this imaging modality based on hypoxia [51, 280] or pH changes [279, 281]. Additionally, molecular probes can be conjugated with fluorescent molecules to visualise molecular-specific alterations of cancer using fluorescence. A successful optical molecular imaging strategy must use an agent which can be safely and effectively delivered to target tissue *in vivo*. The agent should also be able to provide tissue images in real time with the desired spatial resolution and field of view, with large signal to background ratios (SBRs). It is clearly useful to detect changes at the cellular and molecular level rather than rely on anatomical characteristics alone which are commonly the case at present [272]. Tumours may be able to be characterised without biopsies or surgery, and allow for accurate staging, re-staging and drug response monitoring, paving the way towards true personalised medicine [272]. Molecular imaging modalities may also be used for intraoperative surgical guidance and evaluation of surgical margins, thus improving outcomes [272].

9.5.2.1 Light-Induced Fluorescence from 5-Aminolevulinic Acid (5-ALA)

5-ALA is a physiological precursor of fluorescent photosensitizer protoporphyrin IX (PpIX) which is involved in biosynthesis of heme. In aqueous solution, 5-ALA can readily penetrate the cells with abnormal keratin, like those found in oral neoplastic lesions. The excess of 5-ALA results in accumulation of intracellular porphyrins, especially PpIX, which results in increased tissue fluorescence. Irradiation of the lesion with visible light of 405 nm leads to red fluorescence and the difference in red fluorescence of normal and abnormal oral mucosa helps in discrimination between malignant and non-malignant tissues. Figure 9.6 showcases the increased red fluorescence due to higher levels of PpIX in areas of malignancy in a hamster following topical application of 5-ALA.

Leunig et al. [282] studied the utility of 5-aminolevulinic acid–induced protoporphyrin IX fluorescence for the detection of squamous cell carcinoma of the oral cavity. Fifty-eight patients with suspected oral cancer received topical application of 0.4 % 5-ALA. They noticed high intensity of fluorescence in neoplastic tissue as

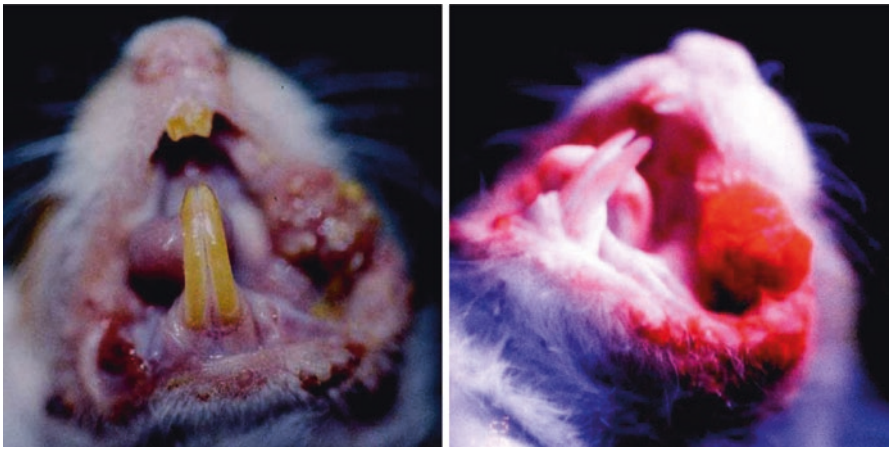

Fig. 9.6 Fluorescence investigation 3 h after topical application of 5-ALA. *Left*- Visible light (λ : 400 ~ 750 nm) image detailing anatomy. *Right*- *Blue* light (λ : 380 ~ 420 nm) image demonstrating increased red fluorescence in areas of malignancy of hamster cheek pouch (Image provided by Dr. Petra Wilder-Smith)

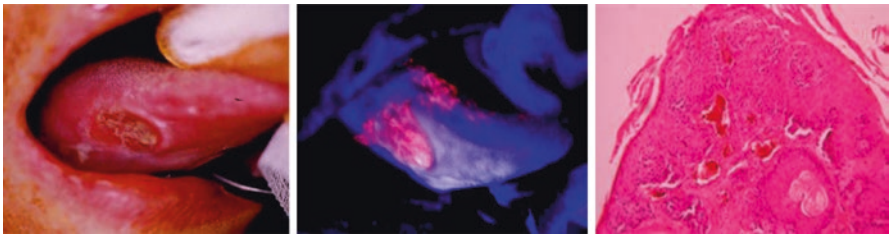

Fig. 9.7 Clinical *in vivo* imaging of squamous cell carcinoma PpIX fluorescence after topical application of 5-ALA. *Left*- *White* light image for registration purposes. *Middle*- PpIX *red* fluorescence observed in areas of malignancy. *Right*- Histology to confirm presence of squamous cell carcinoma (Image provided by Dr. Petra Wilder-Smith, taken from: Chang et al. [283])

compared to surrounding normal tissue. Biopsy evaluation revealed specificity of 60 % and sensitivity of 99 % for detection of oral squamous cell carcinoma by topical application of 5-ALA. In a more recent study done by Chang et al., topical application of 5-ALA at a concentration of 2.5 mg/ml was applied *in-vivo* and the resulting fluorescence was observed 3 h later [283]. As observed in Fig. 9.7, light-induced fluorescence detection using topical Photofrin provides a sensitive, non-invasive technique for the early identification of malignant neoplasms in the oral cavity.

Similarly, Sharwani et al. studied fluorescence imaging after topical application of 5-ALA as a way to improve detection of various oral tissue pathologies. Seventy-one patients with clinically suspicious oral leukoplakia were treated with 5-ALA oral rinse prior to fluorescence imaging. Following this, a surgical biopsy was performed from the examination site and the results of the fluorescence spectroscopy

were compared with the histopathological results. The authors reported an increase in red-to-green fluorescence in dysplasia and carcinoma in situ with a sensitivity of 83–90 % and a specificity of 79–89 % [284]. Fluorescence spectroscopy of oral mucosa following topical application of 5-ALA has great potential as a non-invasive method for the detection of early dysplastic and malignant lesions.

9.5.2.2 Fluorescent Glucose Metabolism

Malignant cells also have increased glucose metabolism which is related to the over expression of Glut-1 (Glucose transporter protein type 1) and HK II (hexokinase II). F18 Fluorodeoxyglucose (FDG) PET imaging is the well-established technique of utilising molecular imaging for assessing tumour glucose metabolism. This technique, however, is limited by poor spatial images, especially for early lesions. Another limitation is that the detection limit of PET is in the magnitude of 10,000,000 cancerous cells, which corresponds to a lesion diameter of at least 1 mm. In a clinical setting, the detection limit is about 10 times greater due to factors like respiratory motion artifacts [285]. For these reasons, there is a very limited role of PET in early detection of oral cancers.

Optical imaging of glucose metabolism is an alternative technique for utilising tumour glucose metabolism for early detection of oral neoplasia. The technique involves the application of fluorescently labelled deoxyglucose to oral mucosa, fresh biopsy specimens and/or resected tumors and evaluating the resulting fluorescence. This technique not only gives wide-field images of large areas but also allows targeted high-resolution imaging of areas of interest. Nitin et al. evaluated the fluorescent contrast properties of topically applied 2-NBDG (2-[N-(7-nitrobenz-2-oxa-1,3- diazol-4-yl)amino]-2-deoxy-D-glucose) in freshly resected clinical specimens of normal and neoplastic oral mucosa using both high resolution confocal, real-time microendoscopic and wide-field fluorescence imaging [286]. They concluded that 2-NBDG can be used by both imaging techniques making it a unique topical agent for early detection of oral neoplasia. In high grade dysplasia and cancers, 2-NBDG was taken up by neoplastic cells throughout the lesion as compared to limited uptake in basal epithelial cells in normal epithelium. Mean fluorescence intensities of neoplastic tissue were about 3.7 times higher than non-neoplastic tissue. There was about a 30 times increase in fluorescence after labelling with 2-NBDG as compared to normal samples, highlighting its potential in early cancer detection.

9.5.2.3 Evaluating Epidermal Growth Factor Receptor (EGFR) Expression

Alterations in cell surface molecular signatures of cancer can also be targeted using exogenous fluorescent molecules. Epidermal growth factor receptor (EGFR) is a known biomarker for the detection of oral neoplasia as well as many other cancers. EGFR can normally be detected in proliferating cells but is markedly overexpressed in rapidly proliferating cells of dysplastic and cancerous tissues leading to uncontrolled growth and survival of malignant cells. In a study by Ang et al., EGFR expression was a strong independent predictor of overall survival, disease free

survival and local relapse [287]. Nitin et al. evaluated the potential of topically applied optical contrast agent to image EGFR expression for early detection of oral neoplasia [288]. The authors noticed that EGF-dye can be uniformly delivered throughout the oral epithelial with depth of penetration greater than 500 μm. Oral neoplasias were reported to produce a 1.5–6.9-fold increase in fluorescence as compared with normal mucosa with both wide-field and high-resolution imaging. This demonstrates the potential of using EGF-targeted fluorescent agents for not only *in vivo* molecular imaging but also allowing real-time detection of tumour margins.

9.5.2.4 Aberrant Glycosylation Via Lectins

Another biomarker of interest includes glycoproteins and glycolipids. Neoplastic transformation is often associated with altered glycosylation of these glycomolecules. Vigneswaran et al. evaluated the alteration of cell surface carbohydrates associated with ordered and disordered proliferation of oral epithelia [289]. The authors concluded that cell surface glycosyl residues are an important component of regulation of cell division and epithelial growth. A prime example of aberrant glycosylation in oral carcinogenesis is the overexpression of sialic acid on cell glycoconjugates [290, 291]. Rajpura et al. showed that sialic acid levels in oral cancer patients was greater than double that of normal patients (63.70 mg/dl versus 30.25 mg/dl, respectively; 41 patients, $p<0.001$) [292]. This has further been verified by Silvia et al. and Joshi et al. who also showed statistically significantly higher values of sialic acid in oral cancer patients [293, 294]. Hyper-/disordered proliferation can be explained by aberrant glycosylation leading to the absence of important terminal residues in cell surface carbohydrates.

In a recent study by Baeten et al., fluorescence molecular imaging of biopsies from seven patients demonstrated that oral squamous cell carcinoma specimens over-expressed sialic acid as compared to the non-neoplastic mucosa [295]. Furthermore, the results illustrated that cell surface changes in sialic acid content of oral neoplasms could be detected with optical imaging using a topically applied lectin (wheat germ agglutinin, WGA, conjugated to a fluorescent molecule). Figure 9.8 demonstrates the molecular specificity of the lectin probe to oral cancer versus normal tissue, which is further substantiated by the ability to diminish signal from the diseased tissue when the lectin probe has been incubated with its inhibitory sugar.

This group has recently performed an *in vivo* clinical trial in which they illustrated the effectiveness of topical WGA-FITC application as a chairside oral cancer diagnostic. The results are pending publication; however, an image of cancer detection using WGA conjugated to a fluorescent molecule (FITC) is shown in Fig. 9.9. Lectin targeting of glycan changes associated with oral carcinogenesis may provide a new avenue for the non-invasive detection and monitoring of oral malignancies, which could be surveyed using optical imaging for immediate chairside results.

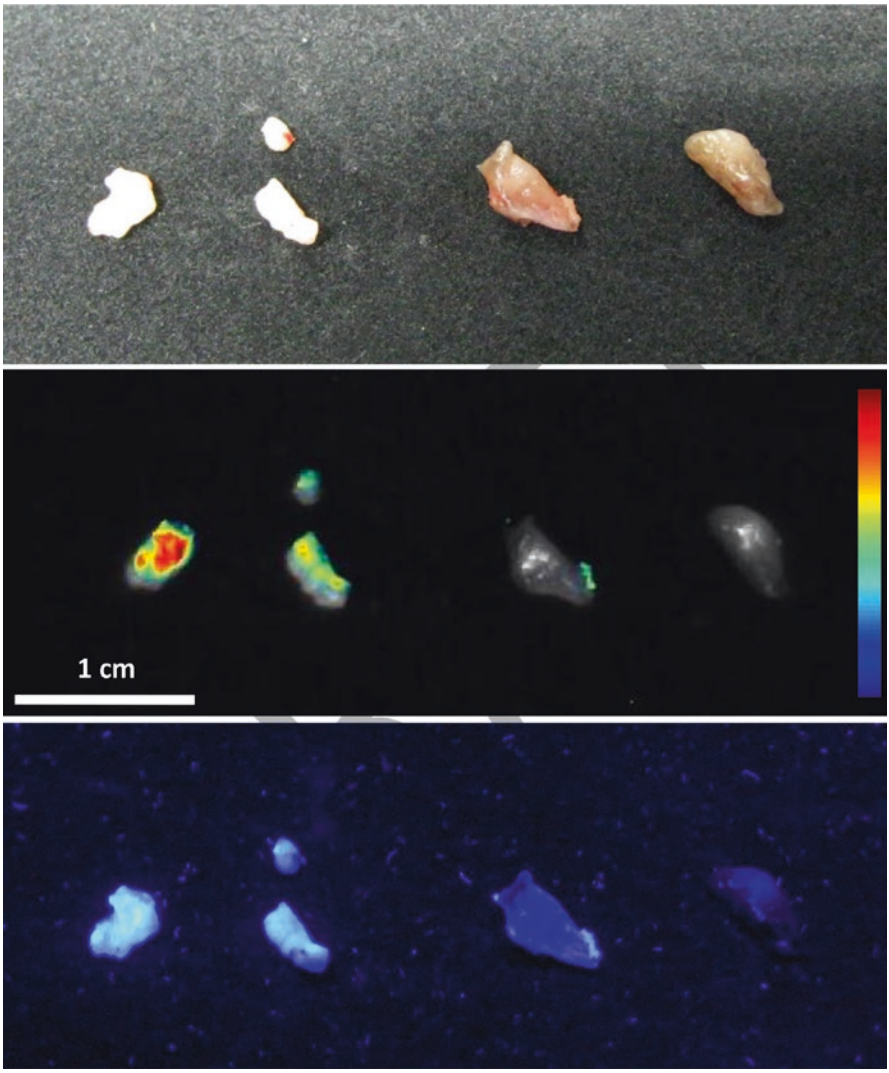

Fig. 9.8 Fluorescence imaging of sialic acid expression in normal and squamous cell carcinoma biopsies taken from the floor of the mouth of a patient demonstrating molecular specificity. *Top-White* light image. *Middle-* Fluorescence image acquired by a scientific CCD camera. *Bottom-* Fluorescence image acquired with digital camera and 450 ± 20 nm bandpass filter. Key (L-R): Cancer biopsy incubated with WGA Alexa Flour 350, cancer biopsy incubated with inhibited WGA Alexa Flour 350, normal biopsy incubated with WGA Alexa Flour 350 and normal biopsy without WGA Alexa Flour 350

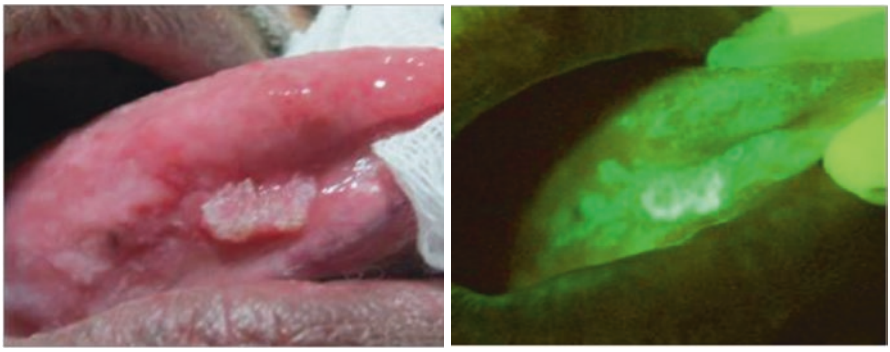

Fig. 9.9 *In vivo* optical molecular imaging of overexpressed sialic acid in a verrucous carcinoma lesion using WGA-FITC. Topical application of WGA-FITC was able to easily detect and delineate the verrucous carcinoma lesion compared to the surrounding tissue. Fluorescent imaging was performed using a filtered blue light (ESPýOC, Visual Solutions, Racine, WI) and a digital camera fitted with an optical filter for FITC visualization. *Left*: clinical image, *right*: WGA-FITC image

Conclusion

Early detection of oral cancer is of high priority due to its significant impact on survival rates. Clinical examination alone does not provide high enough accuracy to effectively downstage oral cancer, as oral precancerous and cancerous lesions share many characteristics with common benign oral lesions (keratinisation, ulceration, inflammation, etc.). Various diagnostic aids and adjunctive techniques are available to complement conventional clinical examination (i.e., visible light examination and palpation of suspicious areas). The efficacy of these adjunctive products and techniques continues to be studied; however, these aids and techniques are likely more useful in screening of high-risk populations with better diagnostic results. Future promise is seen within molecularly specific diagnostic techniques which aim to detect early carcinogenic alterations. Such approaches may reduce the stage of cancer diagnosis, morbidity and costs associated with treatment.

9.6 Key Points

- Oral potentially malignant disorders (OPMD) include leukoplakias, erythroplakias and erythroleukoplakias (commonly referred to as speckled leukoplakias). Although not entirely agreed upon, the link between oral cancer and oral lichen planus or chronic hyperplastic candidosis is being investigated further.
- Early detection and diagnosis of oral neoplastic changes is the best way to improve patient outcomes.

- Conventional oral examination is based on visual inspection under normal white light and palpation of suspicious lesions, usually performed by dentists or physicians. However, the effectiveness and accuracy of conventional oral examination is limited.
- Toluidine blue and reflectance visualisation
  - A variety of diagnostic aids and adjunctive techniques are commercially available, such as toluidine blue, ViziLite™, ViziLite Plus™ and MicroLux/DL.
  - Data indicate that alternative diagnostic techniques can improve diagnostic performance in high-risk populations, but there is a little evidence to support their effectiveness in low-risk populations, outside using white light.
- Optical fluorescence imaging (tissue autofluorescence)
  - The VELscope™ and Identafi® are commercially available adjunctive devices to visualise loss of tissue autofluorescence associated with precancer and cancer in the oral cavity.
  - Digital image processing of wide-field autofluorescence images can be used to outline suspicious regions in real time.
  - The autofluorescence observed in wide-field images of the normal oral mucosa originates primarily from stromal collagen. Oral neoplasia is associated with a loss of stromal autofluorescence.
  - Benign lesions, such as inflammation, are also associated with loss of stromal autofluorescence, which may limit diagnostic specificity especially in low-risk populations.
- Other optical imaging techniques
  - Includes high resolution imaging techniques such as optical coherence tomography, angle-resolved low-coherence interferometry and raman spectroscopy.
  - High-resolution imaging of oral tissue can visualise morphologic and architectural features of the epithelium *in vivo* with subcellular resolution, including the characteristic changes in nuclear size, shape and density associated with oral precancer.
  - High-resolution imaging may provide a tool to discriminate benign changes, such as inflammation, from neoplasia with better specificity than wide-field imaging.
- Combination of wide-field and high-resolution imaging
  - Multimodal optical imaging – a combination of wide-field autofluorescence and high-resolution imaging – may yield the best sensitivity and specificity for detection of oral neoplasia.
  - Particular emphasis should be given to evaluating multimodal optical imaging in a low-risk population.
- Optical molecular imaging using exogenous molecular probes
  - Biomarkers capable of differentiating cancerous and healthy tissues may provide a mechanism to detect oral cancer at its early and most treatable stages.
  - Research using 5-ALA, EGF (or other EGFR binding molecules), fluorescent glucose and fluorescently labelled lectins shows great promise as potential molecular probes for oral cancer detection.

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Conflict of Interest Statement The author declares that he has no personal or financial conflict of interest to declare in relation to the work presented in this chapter. CSF undertakes clinical and laboratory research into oral cancer and pre-cancer early detection, imaging, surgical margin delineation and molecular genomics utilising various technologies listed in this chapter, but has no financial relationship with any of the manufacturers which may bias this work.

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Screening for Oral Cancer

10

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10.1 Introduction

Oral cancer refers to malignant neoplasms arising in the mucosa of the lip, tongue and mouth (International Classification of Diseases 10th Edition (ICD-10) codes C00-06) which are lined by stratified squamous epithelium. More than 90 % of the cancers occurring in the oral cavity are squamous cell carcinomas of varying differentiation, predominantly caused by chronic exposure to tobacco use in any form, alcohol drinking or both and are rarely due to chronic traumatic irritation. However, there is considerable misclassification and overlapping of cancer sites when the generic term "oral cancer" is used such as malignant neoplasms originating in adjacent anatomical sites such as the oropharynx and hypopharynx, even larynx and oesophagus, have been included as "oral cancer" by many authors and reports in the scientific literature, which makes interpretation and comparison of

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oral cancer burden and outcomes somewhat challenging. Our discussion in this chapter refers to cancers occurring in ICD-10 sites C00-C06 as oral cancer. According to the most recent global estimates, oral cancer (C00-06) is the thirteenth most common cancer in the world, accounting for an estimated 300,000 new cases and 145,000 deaths in the world around 2012, of which 200,000 cases and 112,000 deaths occurred in less developed countries [1].

Whereas incidence rates for cancers occurring in the different subsites of oral cavity may be obtained from population-based cancer registries, mortality data are reported together for a number of head and neck cancer sites collectively and not subsite wise, and hence interpretation and comparison of mortality data for oral cancer can be difficult. There is more than tenfold variation in oral cancer incidence rates across the world where the incidence is more than twofold higher in men than women in most regions (Figs. 10.1 and 10.2) [2]. Although largely preventable by avoiding exposure to risk factors such as tobacco and alcohol, a high incidence of oral cancer is observed in the Indian subcontinent, parts of Central and Eastern Europe, France, Southern Europe, South America and Oceania.

The varying risks of oral cancer between countries, regions, populations, men and women largely reflect the differences in the prevalence of betel quid/areca nut/tobacco chewing, tobacco smoking and alcohol drinking habits. The highest incidence of oral cancer in both sexes is observed in South Asia where betel quid chewing is a major risk factor [1, 2]. High-risk groups for oral cancer can be defined on the basis of age, sex, tobacco and alcohol drinking. A recent meta-analysis involving 1885 cases of oral cancer and 2248 controls and 956 cases of oral precancerous lesions and 675 controls concluded a strong association between high-risk HPV, particularly HPV16 and oral squamous cell carcinoma; significant association was found between pooled HPV DNA detection and oral cancer (relative risk (RR): 3.98; 95 % CI: 2.62–6.02); HPV was also associated with oral precancerous lesions (RR: 3.87; 95 % CI: 2.87–5.21), suggesting the rejection of the null hypothesis that HPV is equally prevalent in normal oral mucosa, oral precancerous lesions and oral cancer [3]. Another recent meta-analysis reported a 25.3 % prevalence of HPV 16/18 infection in 186 tissue samples of dysplastic lesions from the oral cavity [4]. Further clarification of high-risk HPV infection in the causation of oral precancerous lesions and cancer in well-conducted epidemiological studies is an important research priority. However, avoiding tobacco use in any form and moderation in alcohol consumption can prevent more than three-fourths of the oral cancer burden globally and, at the same time, offer the principal means to control oral cancer globally.

Advanced oral cancer is functionally one of the most debilitating and cosmetically disfiguring cancers. Despite the many advances in diagnosis and treatment, the overall 5-year survival rate for oral cancer has been more or less constant over the last 40 years, as more than 70 % of oral cancers are still diagnosed in locally advanced stages in many countries [5–7]. Overall, less than 40 % of patients with oral cancer survive for more than 5 years; the 5-year survival ranges from around 90 % for patients with localised, stage I cancer to less than 30 % for those with disease involving regional lymph nodes and less than 5 % for those with distant metastases [5–9]. Treatment of locally advanced disease often produces distortions

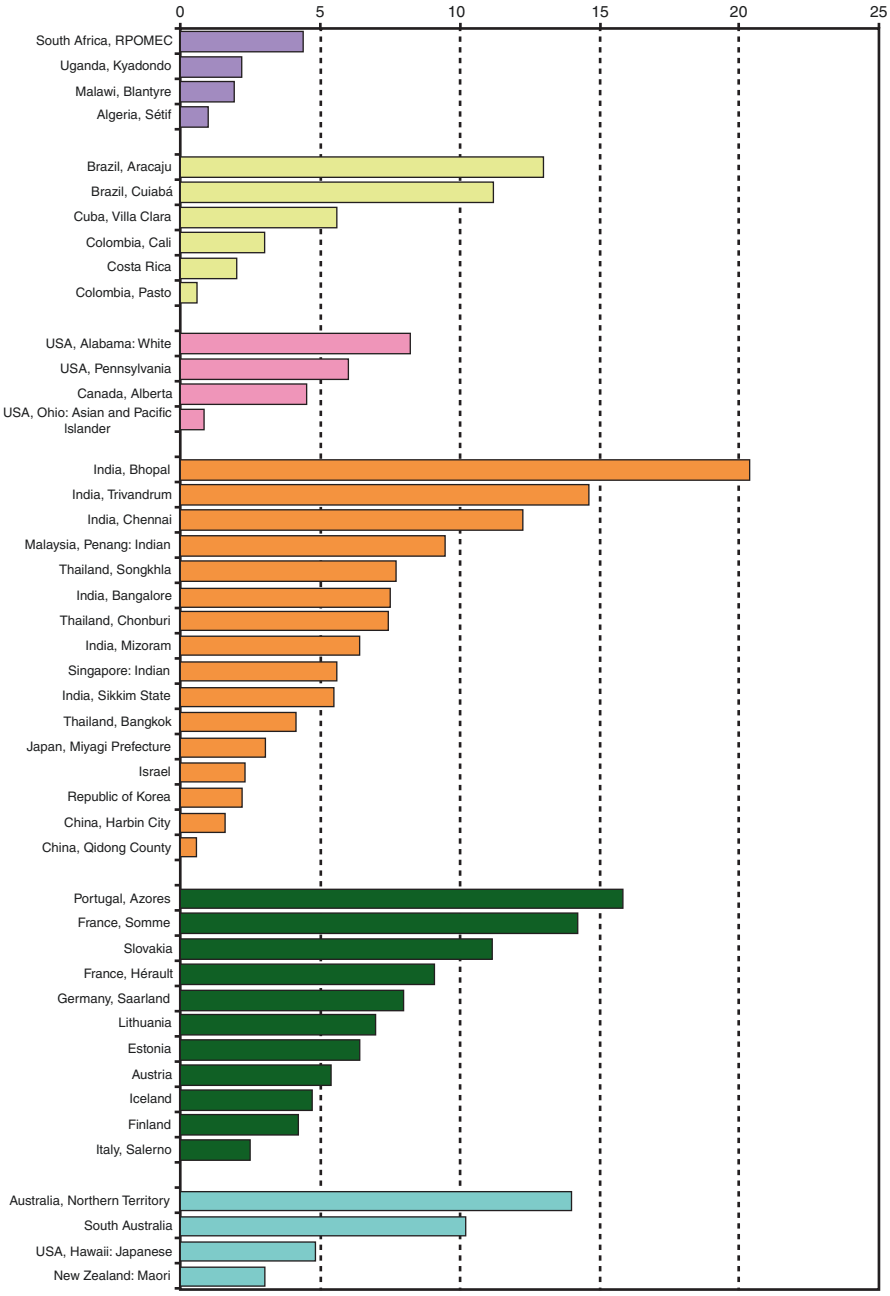

Fig. 10.1 Age-standardised incidence rates of oral cancer (C00-06) in men in selected populations in five continents (2003–2007) [2]

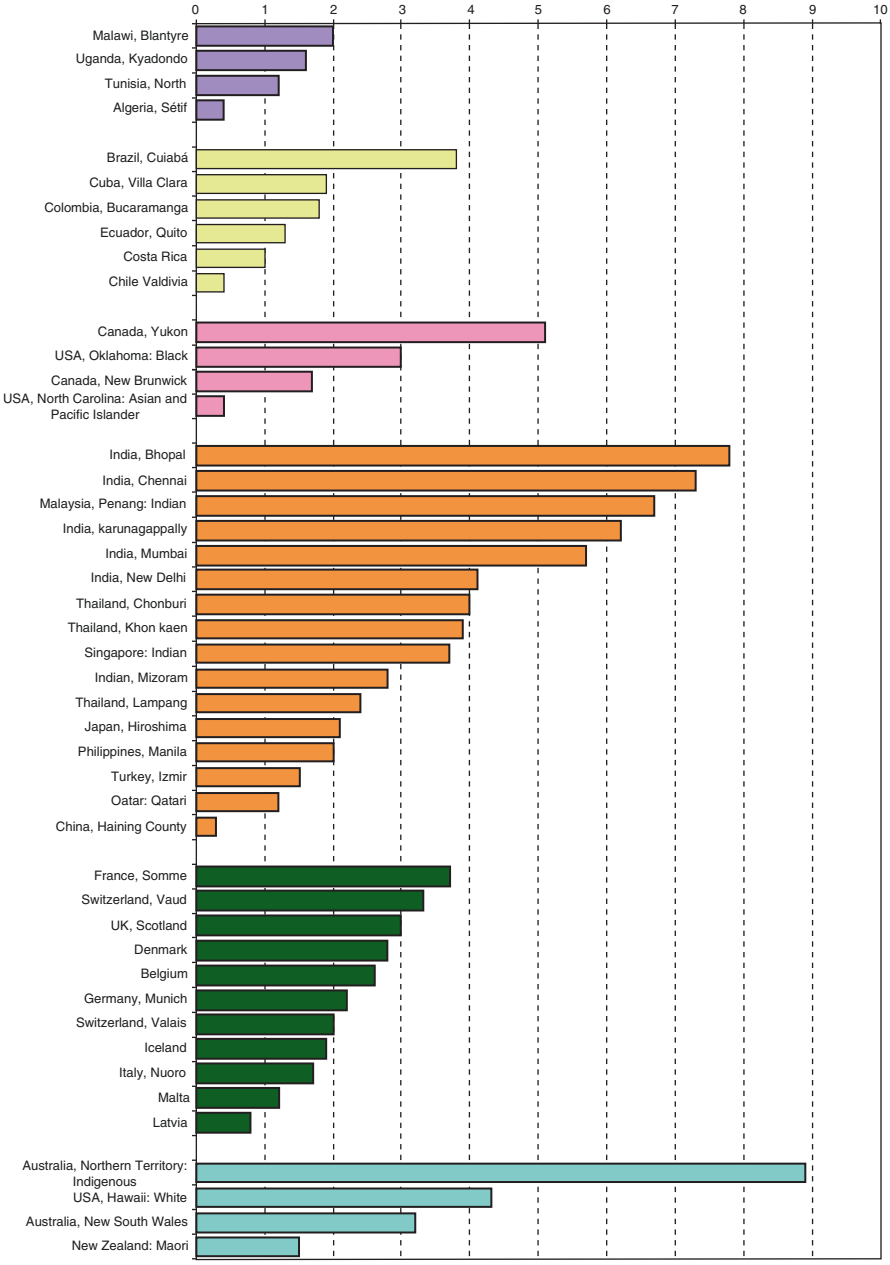

Fig. 10.2 Age-standardised incidence rates of oral cancer (C00-06) in women in selected populations in five continents (2003–2007) [2]

in cosmetic appearance and difficulties in mastication, speech and swallowing, leading to difficulties in social interactions.

Secondary prevention by screening offers the principal means of diagnosing early-stage disease and reducing oral cancer mortality. Screening involves testing large numbers of asymptomatic individuals with a simple, safe and affordable test to identify those with early, preclinical asymptomatic disease of interest. The suitability of oral cancer for screening, different approaches for screening, and the evidence for the effectiveness of oral cancer screening as a control option and the potential benefits, harms and limitations of screening for oral cancer are briefly discussed in this chapter.

10.2 Screening as a Disease Control Intervention

A decision to introduce population-based screening should take into account evidence on feasibility, acceptability, safety, effectiveness and cost-effectiveness of reducing disease burden from appropriately designed and conducted population-based studies. Screening involves the application of a relatively simple, inexpensive test to a large number of apparently healthy people in order to identify those who have preclinical disease or are likely to develop the disease in the future. A disease is considered suitable for screening if it is an important public health problem and has a long natural history and a long detectable preclinical phase, and effective treatment is available if detected early. In addition, an appropriate, suitable, feasible, affordable, easy to use, safe, acceptable and accurate screening test should be available to detect the disease early.

A potential benefit from screening can be expected if the disease has a long and detectable preclinical phase, and, in the absence of intervention, most patients with preclinical disease progress to symptomatic clinical disease, and if effective and affordable, treatment is available for the preclinical disease. However, screening is of limited benefit if most of the preclinical cases do not progress to overt clinical disease, if most of the clinical cases do not go through a detectable preclinical phase, if preclinical cases have a very short detectable phase or if no effective and affordable treatments are available.

Screening programmes may be organised or unorganised. Organised programmes are characterised by centralised screening invitations to a well-defined target population, systematic recall, diagnostic investigations, treatment and follow-up care of persons found with abnormalities on screening, centralised quality assurance and a constantly updated screening information system with linkage to other information systems such as cancer registries and death registration systems for monitoring and evaluation of the programme. In unorganised programmes, screening tests are provided to individuals on request or opportunistically during their routine healthcare interactions with doctors. Organised screening programmes for cancers such as cervical cancer have shown the greatest effect while using fewer resources than unorganised programmes. The critical components of successful screening programmes are high coverage of target population with accurate, quality-assured screening tests and

of screen-positive persons with diagnostic investigations, treatment and follow-up care, which are most cost-effectively met within organised screening programmes.

Screening programmes are evaluated by a set of process and outcome measures. The process measures include the number of people screened, proportion of target population screened, number of times screened, number of people positive on screening tests, number of screen-positive people undergoing diagnostic investigations and treatment, number of people diagnosed with preclinical disease, positive predictive value of the screening test, total costs of the programmes and cost per year of life saved. The outcome measures used to assess the success of screening programmes include incidence rate of advanced disease, stage distribution, case fatality and disease-specific death rate in the population invited for screening, cost-effectiveness of screening, safety and quality of life. Quality of life issues have seldom been addressed in the context of cancer screening programmes, in spite of their appeal and importance.

10.3 Oral Cancer as a Suitable Disease for Screening

Oral cancer is an important oncological problem in many countries and regions. It occurs in one of the most easily accessible anatomical sites of the human body. It has a long and detectable preclinical phase consisting of potentially malignant disorders that may progress to invasive if left unattended and very early asymptomatic invasive cancers presenting as painless, small ulcers, nodular or granular lesions or growths. Visual screening of the oral cavity, with tactile palpation, is a simple, non-invasive, acceptable, affordable, safe, feasible and accurate screening test. The malignant transformation of the precancerous lesions can be prevented by appropriate interventions such as preventing exposure to tobacco use and alcohol drinking and, in selected instances, by excision of the lesions. Early, localised oral cancers measuring less than 2 cm with no spread to the regional lymph nodes can be effectively treated and cured with single-modality treatments, such as surgery or radiotherapy with no significant functional or cosmetic defects, resulting in 5-year survival rates exceeding 90 %. Thus, oral cancer satisfies all the criteria for a suitable disease that can be controlled by screening.

The target population for oral cancer screening are those aged 35 years and above and those who use tobacco or alcohol in any form. Oral cancer incidence is rare below 35 years (Fig. 10.3) [2]. More than 90 % of oral cancers occur in persons with tobacco and/or alcohol habits. Targeting these high-risk individuals enhances cost-effectiveness of screening.

10.4 Objectives of Oral Cancer Screening

The primary aim of oral cancer screening is to prevent deaths from oral cancer and improve functional outcomes such as cosmetic appearance, deglutition, mastication, taste perception abilities and phonation leading to a better and

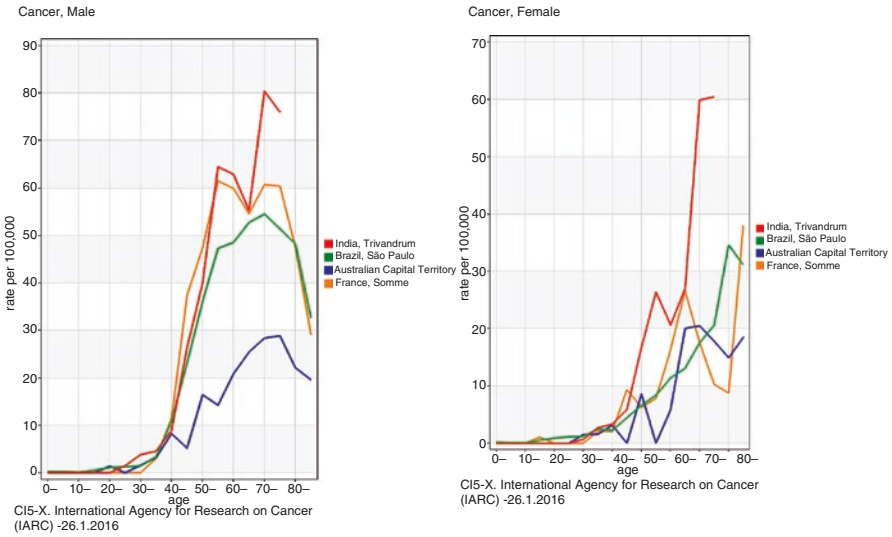

Fig. 10.3 Age-specific incidence rates of oral cancer in selected populations (2003–2007) [2]

acceptable quality of life in a cost-effective manner. To fulfil these objectives, oral cancer must be identified in the preclinical phase; treatment must be more effective in the preclinical phase than in the symptomatic phase and must reduce the rate of death from oral cancer; the screening test must be affordable and safe and must have a high sensitivity and specificity with a low frequency of false-positive tests.

10.5 Oral Potentially Malignant Disorders (OPMDs)

Oral potentially malignant disorders (OPMDs) denote clinically obvious morphologically altered oral mucosa in which cancer is more likely to occur and may show dysplasia or carcinoma on histological examination. A number of OPMDs have been described which include leukoplakia, erythroplakia, lichen planus, submucous fibrosis, palatal lesions in reverse smokers and actinic keratosis, among others [10, 11] (Fig. 10.4). Most OPMDs can be visually detectable due to their more or less distinct clinical signs. A significant proportion of clinically detected lesions resembling OPMD harbour underlying preclinical invasive cancer. Hence visual inspection and palpation of the oral mucosa are of high relevance in the prevention and early diagnosis of oral cancer.

The estimated annual malignant transformation frequency of OPMD ranges between 0.13 and 2.2 % [12, 13]. A higher risk of malignant transformation may be associated with the following factors: lesions in women, lesions of long duration, large OPMDs, OPMDs in non-users of tobacco, tongue and floor of mouth lesions, non-homogeneous lesions and lesions showing epithelial dysplasia [12, 14].

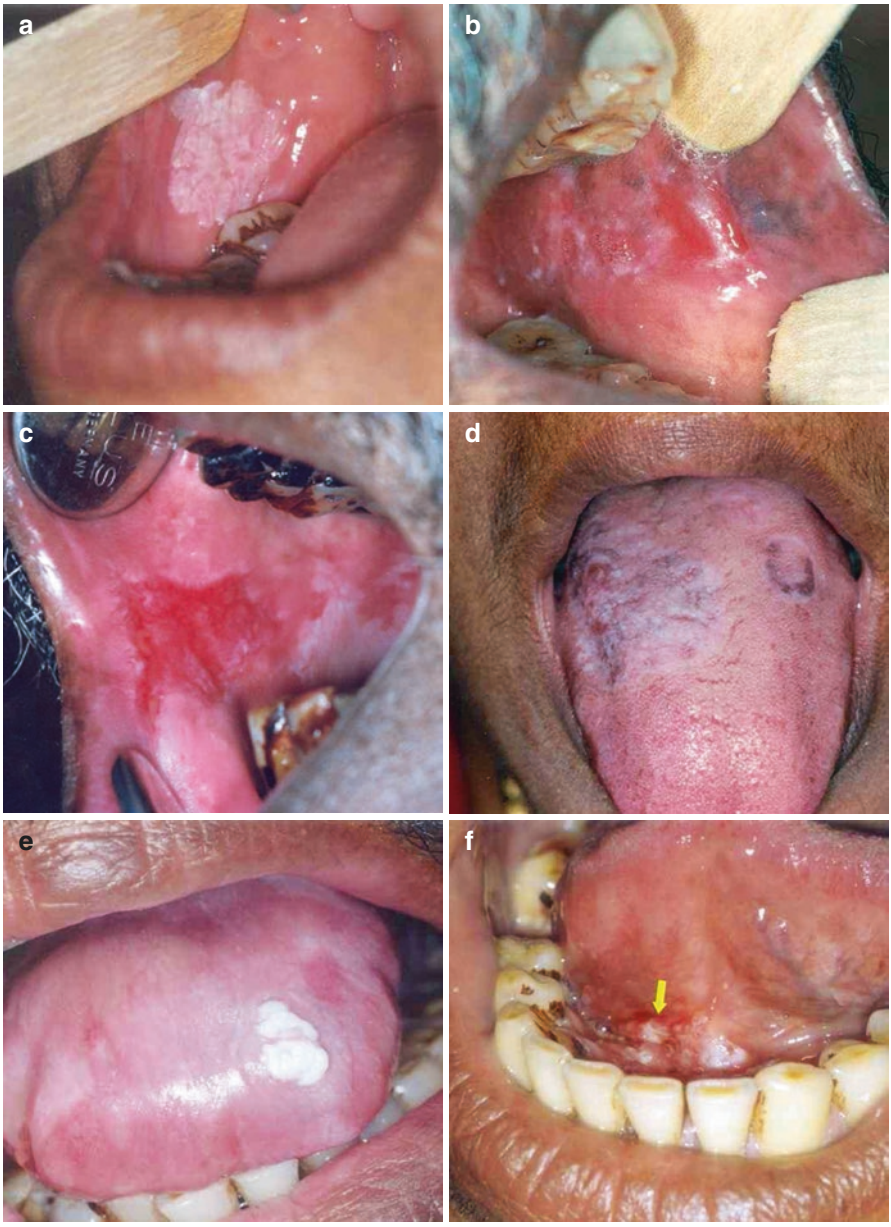

Fig. 10.4 (**a**) Homogeneous leukoplakia on the right buccal mucosa. (**b**) Ulcerated leukoplakia in the left buccal mucosa. Note the ulcerated area in the centre surrounded by white patches at the periphery and the hyperpigmentation anteriorly. (**c**) Erythroplakia. Note the red patch on the right buccal mucosa with white areas posteriorly. (**d**) Lichen planus. Note the white 4×3.5 cm patch on the right side of the dorsum tongue intermingled with areas of pigmentation. Another annular form of lichen planus can be seen on the left side of the dorsum tongue. (**e**) Oral submucous fibrosis of the tongue. Note the coexisting verrucous leukoplakia. (**f**) Early invasive cancer in the floor of mouth. Note the erythematous ulceroproliferative growth on the floor of the mouth (*arrow*)

However, it is currently impossible to predict with certainty which OPMD will turn malignant during follow-up in patients. Availability of accurate biomarkers predicting progression of OPMDs to cancer would enable targeting the OPMDs that have the potential to turn malignant with appropriate treatment and follow-up care. However, there is currently no single or set of clinically relevant and useful biomarkers that can readily predict OPMDs that could progress to invasive cancer in the absence of intervention [15].

Avoiding tobacco use and alcohol drinking is the most effective way to prevent the progression and reduce the risk of malignant transformation of OPMDs. There is no evidence to support multivitamin, vitamin A and beta-carotene supplementation in the management of OPMDs, to prevent further progression. Chemoprevention is not yet a clinically proven option, and the currently available level of evidence would not permit routine and long-term use of any chemopreventive agent in clinical practice [16]. Although cold knife excision or laser ablation/excision may be attempted to excise small localised, accessible OPMDs, recurrences and progression may still occur because of the entire oral mucosa being susceptible to carcinogenesis due to the field cancerisation effect of the previous exposure to carcinogens [17–19]. Discontinuation of tobacco and alcohol use is the critical element in the overall management to prevent progression of OPMDs.

Leukoplakia is defined as a white plaque of questionable risk having excluded other known disorders that carry no increased risk of cancer [10]. Around 2–5 % of tobacco users and alcohol drinkers may have oral leukoplakia. Leukoplakia may be clinically categorised as homogeneous or non-homogeneous types. Whereas homogeneous lesions appear as thin, flat, uniform, smooth white lesions, non-homogeneous leukoplakia may show a variety of clinical appearances described as ulcerated leukoplakia with a white and red appearance; nodular leukoplakia with tiny white pinhead size raised nodules on an erythematous background or as verrucous leukoplakia with proliferative, warty appearance. All clinically diagnosed leukoplakias warrant biopsy to exclude underlying cancer and dysplasia. Histologically, leukoplakia may show the presence or absence of dysplasia of varying grades, although substantial interobserver variations exist in the assessment of dysplasia and its severity. Elimination of risk factors, such as tobacco and alcohol, and follow-up examinations are important in the prevention of further progression. Excision may be advised, if the lesion is of localised nature, irrespective of the presence or absence of epithelial dysplasia, although its relevance in preventing malignant transformation, particularly in the absence of habit intervention, is not clear [17, 20]. The long-term benefit of specific treatments such as surgical excision in terms of preventing recurrences and invasive cancer has not yet been addressed in the context of well-planned studies with long-term follow-up.

Erythroplakia, a relatively uncommon OPMD, presents as a red patch with smooth or granular surface that cannot be characterised clinically or pathologically as any other definable disease [10, 21]. Histologically, erythroplakia has a higher probability than leukoplakia to demonstrate dysplasia or underlying occult invasive cancer and has a higher probability of malignant transformation. All erythroplakias would warrant excision or multiple biopsies in the case of large, diffuse lesions not amenable for complete excision.

Oral lichen planus may present as interlacing white keratotic lines (known as Wickham's striae), with an erythematous border, or as a mix of erythematous and ulcerated areas surrounded by finely radiating keratotic striae. Oral lichen planus migrates over time, tends to be multifocal and often presents with symptoms ranging from episodic pain to severe discomfort.

Oral submucous fibrosis (OSMF) presents with vesiculation, burning sensation, blanching of the oral mucosa and intolerance to spicy food followed by loss of papillae in the tongue, stiffening and atrophy of the oral and pharyngeal mucosa as the disease progresses. Fibrous bands appear in the oral mucosa and faucial pillars and encircle the lips in advanced stages leading to loss of tissue mobility and reduced mouth opening, distorted uvula, woody changes on the buccal and tongue mucosa leading to deglutition and speech problems. Histologically, fibrosis and hyalinisation occur in the lamina propria followed by atrophy of the overlying epithelium, which is susceptible to oral cancer when exposed to carcinogens. The occurrence of OSMF is restricted to people of Indian subcontinent origin. Early OSMF may be a reversible condition by elimination of risk factors such as tobacco and alcohol, as evidenced by the disappearance of mucosal blanching and regeneration of tongue papillae.

Palatal lesions are specific to populations who smoke with the lighted end of tobacco product inside the mouth (known as reverse smoking), resulting in white or mixed reddish-white lesions of the palate. Actinic keratosis is clinically characterised by ulcerative, crust forming lesions on the labial mucosa along the vermillion border; histological examination may show hyperkeratosis with or without epithelial dysplasia.

10.6 Invasive Oral Cancer

Early invasive oral cancer may present as small proliferative growths in red or white lesions, as nodular or red and white patches and as ulcerating and proliferative growths and persistent ulcers (Fig. 10.4). Locally advanced cancers present as large ulcerating and proliferative growths, often involving adjacent tissues, bone, skin and with cervical lymph node enlargement. Early, localised oral cancers measuring less than 2 cm, without regional lymph node involvement, may be treated with single-modality treatment with surgery or radiotherapy and have cure rates exceeding 90 %, whereas locally advanced diseased, with or without regional lymph node metastasis, would require combined modality treatment involving surgery, radiotherapy and chemotherapy, with often unsatisfactory survival and cosmetic/functional outcomes [5–7].

10.7 Screening Tests for Oral Neoplasia

10.7.1 Visual Screening by Doctors and Health Workers

The tests used for oral cancer screening are given in Table 10.1. Visual screening and tactile palpation under bright light are the most widely used tests for the early detection of OPMDs and early asymptomatic, preclinical cancers. It involves

Table 10.1 Oral cancer screening methods

Oral visual screening
Mouth self-examination
Adjuncts to visual screening
Toluidine blue intravital staining
Chemiluminescence (ViziLite™, MicroLuX DL™, Orascoptic DK™, etc.)
Autofluorescence (VELscope™)
Autofluorescence spectroscopy
Exfoliative cytology (OralCDxBrush Test™)
Saliva analysis

systematic visual and physical examination of the intra-oral mucosa under bright light, for signs of OPMDs as well as early oral cancer, followed by careful inspection and digital palpation of the neck for any enlarged lymph node masses. It is a provider dependent, subjective test and hence its performance in detecting lesions varies between different providers. Comprehensive knowledge of the oral anatomy, natural history of oral carcinogenesis and the clinico-pathological features of the OPMDs and preclinical cancers are important prerequisites for an efficient provider of oral visual screening. A variety of healthcare personnel such as dentists, general practitioners and specialists, such as oncologists, surgeons, nurses and auxiliary health workers, may provide oral visual screening after training. A variety of resources are available to gain competency in oral visual screening (digital atlas: http://screening.iarc.fr/atlasoral.php).

The sensitivity of oral visual inspection to detect OPMDs and cancer lesions varied from 40 to 93 % and the specificity ranged from 50 to 99 % in different studies, indicating that visual screening is a suitable screening test for oral neoplasia [22–28]. The positive predictive value for OPMDs and oral cancer ranges from 2 to 20 %, depending upon the prevalence of lesions, the sensitivity of the test and competency of the provider. The potential harms of oral visual screening may include additional diagnostic investigations such as incisional or excisional biopsy and anxiety associated with false-positive screening tests; detection and treatment of biologically insignificant, nonprogressive OPMDs that may have no impact on oral cancer mortality; and false reassurance from false-negative tests.

Oral visual screening has been implemented in national oral cancer screening programme settings in countries such as Cuba and Taiwan and is the most widely used clinical approach for early detection of oral cancer. A significant reduction in the risk of advanced oral cancer was observed in a case control study of oral visual screening in the context of the Cuban national oral cancer screening programme [29]. A randomised controlled trial is the most unbiased means to evaluate whether a screening test reduces disease-specific deaths or is considered as the gold standard when evaluating the efficacy of screening tests. Among all oral cancer screening tests, evidence for the efficacy and cost-effectiveness in reducing oral cancer mortality in the context of a randomised trial has only been addressed for visual screening [30, 31, 53]. A significant 34 % reduction in oral cancer mortality among the high-risk group of tobacco or alcohol users in the general population following three rounds of oral visual screening has been demonstrated in a cluster randomised

controlled trial [30, 53]. A cost-effectiveness study in the context of this trial established that oral cancer screening by visual inspection was performed for under US$6 per person and the incremental cost per life-year saved was US$835 for all individuals eligible for screening and US$156 for tobacco or alcohol users, indicating that the most cost-effective approach is to screen for oral cancer by visual inspection in high-risk populations of tobacco and/or alcohol users [31]. Long term follow up in this study demonstrated sustained reduction in oral cancer mortality, with larger reductions in those attending repeated screening rounds [53]. Although mouth self-examination using a mirror has been evaluated as a screening test in some studies [32, 33], whether it could lead to reduced oral cancer mortality is not known.

10.7.2 Mouth Self-Examination

The utility of mouth self-examination (MSE) in improving the awareness of oral cancer and its risk factors and its feasibility and performance as an oral cancer screening tool have been evaluated in studies in India [32, 34]. MSE involves educating and empowering individuals to self-inspect oral cavity using mirrors and look for early changes suggestive of OPMD and oral cancer. In an earlier study involving 22,000 subjects, MSE resulted in the diagnosis of 85 people with OPMD and 7 with oral cancer [32]. In a recent study, MSE lead to the detection of 216 cases of OPMD and 2 of oral cancer from among 34,766 individuals; its sensitivity and specificity were 18 % and 99.9 %, respectively [34]. Although educational efforts on MSE may improve awareness, it is unlikely that MSE as a stand-alone approach will lead to reduction in oral cancer mortality.

10.7.3 Adjunctive Tests to Visual Screening

A variety of techniques (Table 10.1) have been investigated as adjuncts to the standard visual and tactile oral examination under incandescent light to assess if they can improve the distinction between normal and abnormal oral tissues, between benign and dysplastic/malignant changes, over and above that of visualisation using bright light, and if they can lead to the detection of OPMD and cancers that are not visible to the naked eye. The results of their reported accuracy in detecting lesions cannot be extrapolated to the general population as these were based on small case series with high prevalence of lesions. The evaluation of the feasibility, clinical utility as triaging tests and cost-effectiveness of using these adjunctive tests over and above routine visual inspection and palpation and their effectiveness in reducing oral cancer mortality would require well-planned, large-scale population-based studies. Since these tests are more likely to be used as triaging tests, following a positive visual screen, robust assessment of their false-negative rates in well-conducted longitudinal studies with long follow-up is critical.

Toluidine blue (TB) staining, which is believed to stain nuclear acids, has been used to guide biopsies in sites more likely to harbour dysplasia or occult malignancy in oral

lesions [35–38]. It is applied by a swab or as a mouth rinse, with a single application at baseline, or as two applications 14 days apart, and the lesions taking up the blue stain are categorised as positive from where biopsies are directed. Many studies evaluating TB in the past had significant limitations due to variability in the methods and interpretation of test results. It may prove useful in detecting carcinomas in OPMDs, but it is a poor predictor of dysplastic lesions and the high proportion of false-positive stains limits its use as a primary screening test and in primary care settings. A major limitation of the test is that it cannot identify or predict risk of progression for epithelial abnormalities that cannot be seen with naked eye [35, 37]. At present, TB may be used as an adjunct to visual screening to detect carcinomas in OPMD, as it may stain visible lesions with high-risk molecular patterns and could predict risk in cases where little or no microscopic evidence of carcinoma exists [36, 39].

Light-based adjunctive detection tests, based on chemiluminescence such as ViziLite™, tissue fluorescence imaging such as VELscope™, and tissue fluorescent spectroscopy rely on the differential absorption and refraction profiles of different types of light energy due to mucosal structural and metabolic changes following carcinogenesis. The chemiluminescence method involves the use of an oral rinse with 1 % acetic acid for 1 min followed by the examination of the oral mucosa under diffuse chemiluminescent blue and white light at a wavelength of 490–510 nm. The abnormal oral tissue appears acetowhite with bright, sharp and distinct margins and the normal tissue as blue, once the acetic acid removes the glycoprotein barrier and slightly desiccates the oral mucosa [35, 37, 40, 41]. With tissue fluorescence imaging, normal oral mucosa emits a pale green autofluorescence, whereas abnormal mucosa appears darker with respect to the surrounding healthy tissue; autofluorescence spectroscopy that produces lights of various excitation wavelengths and receives and records the spectra of reflected fluorescence from the tissue has also been evaluated in the early detection of oral cancer [35, 37, 42, 43]. These methods have been studied as diagnostic aids in small case series of patients with high frequency of dysplastic and malignant conditions (18–88 %) and have not been evaluated in general population studies with low prevalence of OPMD or by primary care providers. Further well-planned, large, general population-based studies are needed to evaluate and assess whether the overall detection of OPMD and occult cancer is significantly improved by the light-based adjunctive tests and for recommendations concerning their wider clinical utility in routine screening settings.

Exfoliative cytology is used to investigate mucosal abnormalities that would otherwise not be subjected to biopsy due to low-risk clinical features [35, 37, 44]. A number of analytical methods are available for studying cytology specimens. OralCDx™ is a commercially available oral cytology test in which a specially designed brush is used to collect cells that are fixed on a glass slide, stained with a modified Papanicolaou method and analysed microscopically via a computer-based imaging system. Results are reported as negative, atypical or positive. Current evidence indicates that OralCDx™ is accurate in detecting dysplastic changes in high-risk mucosal lesions clinically suggestive of malignancy, but when used in a low-risk population with low prevalence of OPMDs, the accuracy is

reduced [35, 37, 44]. Fine needle aspiration cytology (FNAC) is useful in evaluating clinically suspicious cervical masses suggestive of suspicious involvement of lymph nodes [45].

A recent study concluded that VELscope, OralCDx and toluidine blue staining have high false-positive rates when used to screen routinely for oral cancer [46]. It would be inefficient to allocate scarce healthcare resources to the routine use of these devices for routine oral cancer screening. These devices may be useful as diagnostic triaging devices in specific situations or in cancer referral clinics when the pretest probability of oral cancer is likely to be above 10 %.

The fact that salivary composition is altered in patients with oral cancer, and the permanent contact between saliva and the mucosa, has stimulated interest in investigating salivary analysis as non-invasive oral screening test. This approach relies on measuring specific salivary macromolecules, proteomic or genomic targets such as enzymes, cytokines, growth factors, metalloproteinases, endothelin, telomerase, cytokeratins, mRNA's and DNA transcripts [47, 48]. Well-designed studies are needed to address whether salivary analysis could prove to be a feasible, accurate and cost-effective primary or adjunctive screening tool for oral cancer.

10.8 Biopsy and Histopathology

The reference diagnostic investigation ("gold standard") for oral mucosal lesions that are suggestive of OPMD or cancer is tissue biopsy and microscopic examination. Hence, a biopsy should follow all cases of visually detected OPMDs and suspected malignant lesions as a diagnostic procedure to establish a definite final diagnosis. Biopsy specimens may be obtained using punch, incisional or excisional techniques under local anaesthesia, and the choice of the technique will depend upon several factors. Scalpel biopsy is the most widely accepted technique and the one that shows fewer limitations for obtaining samples from the oral cavity; complete excision will be considered when the size and location of the lesion allows. The accurate histological characterisation and diagnosis of OPMD and malignant oral lesions depend on the quality of the biopsy, adequate clinical information, processing of the specimen and correct microscopic interpretation of the histological patterns. In order to obtain a good-quality oral biopsy, the clinician should avoid crushing the sample with the tissue-holding forceps, infiltrating anaesthetic solution within the lesion, using an insufficient volume of fixing solution and taking insufficient amount of tissue in extension and depth. The specimen should be handled gently, avoiding any crushing, and introduced in the fixing solution such as 10 % formalin. A detailed description accompanying the tissue specimen that describes the identification of the patient, description of symptoms, clinical findings and a probable clinical diagnosis, as well as the orientation of the sample, is mandatory to help the pathologist arrive at the accurate final diagnosis. An explanatory diagram of the biopsy area may be useful for this purpose.

10.9 Evidence for Effectiveness of Oral Cancer Screening

Currently, evidence in terms of efficacy and cost-effectiveness in reducing oral cancer mortality in the context of a randomised trial is available only for visual screening (Tables 10.2 and 10.3, Fig. 10.2) [30, 31, 53]. Subjects aged 35 years and above in 13 clusters in Kerala, India, were randomised to receive three rounds of oral visual inspection at three-year intervals by trained health workers (7 clusters, 96,517 subjects) or to a control group (6 clusters, 95,536 individuals). In all, 33,343 subjects received a single screen, 24,210 two screens and 29,102 three screens in the intervention group (87,645 (91 %) eligible subjects were screened at least once) and overall 5145 were screened positive, of whom 3218 (63 %) complied with referral for diagnosis [30, 53].

The overall and stage-specific case fatality was lower in the intervention group as compared to the control group (Table 10.2). There were 77 oral cancer deaths recorded among all trial participants in the intervention group compared with 87 oral cancer deaths in the control group yielding a mortality ratio of 0.79 (95 % CI: 0.51–1.22) (Table 10.3) [30, 53]. As expected, 96 % of all oral cancer cases and

Table 10.2 Vital status of oral cancer patients by stage and by study group in a randomised trial of oral visual screening, Trivandrum, India, 1996–2004 [30, 53]

	Intervention group		Control group	
Stage	Alive	Dead	Alive	Dead
I	51	5 (9.8)	20	6 (30.0)
II	34	8 (23.5)	17	3 (17.6)
III	37	14 (37.8)	35	17 (48.6)
IV	67	42 (62.7)	70	47 (67.1)
Unknown	16	8 (50.0)	16	14 (87.5)
Total	*205*	*77 (37.6)*	*158*	*87 (55.1)*

Table 10.3 Oral cancer mortality in all eligible subjects and in eligible subjects with tobacco and/or alcohol habits in a randomised trial of oral visual screening, Trivandrum, India, 1996–2004 [30, 53]

	Intervention group	Control group	Rate ratio (95 % CI)
All eligible subjects (35 years old and above)			
Person-years	469,089	419,748	–
Oral cancer cases	205	158	–
Oral cancer deaths	77	87	–
Oral cancer mortality rate/100,000 person-years	16.4	20.7	0.79 (0.51–1.22)
Eligible subjects using tobacco and/or alcohol			
Person-years	234,405	187,281	–
Oral cancer cases	190	156	–
Oral cancer deaths	70	85	–
Oral cancer mortality rate/100,000 person-years	29.9	45.4	0.66 (0.45–0.95)

CI confidence interval

deaths in the study occurred among users of tobacco and/or alcohol, and hence the oral cancer mortality rates among these high-risk subjects in the intervention and control group were compared. There were 70 oral cancer deaths among 45,651 subjects with tobacco/alcohol habits in the intervention group and 85 oral cancer deaths among 38,539 such subjects in the control group, yielding an oral cancer mortality rate ratio of 0.66 (95 % CI: 0.45–0.95) (Table 10.3) [30, 53]. Results of long-term follow-up (15 years) from this trial indicated sustained oral cancer mortality reduction in user of tobacco or alcohol or both [53].

While the study lacked the power to detect significant mortality differences in the general population (people with and without habits) due to the very low risk of oral cancer among those with no tobacco/alcohol habits, the significant 34 % reduction on oral cancer mortality among users of tobacco or alcohol in the intervention group, compared with the control group, clearly established that oral visual screening can reduce oral cancer-specific mortality among people at risk [30, 53]. A cost-effectiveness analysis of this trial reported that oral visual inspection was performed for under US $6 per person and the incremental cost per life-year saved was US $835 for all individuals eligible for screening and US $156 for users of tobacco or alcohol, indicating that the most cost-effective approach to oral cancer screening by visual inspection is to offer it to the users of tobacco or alcohol or both [31]. The findings also support that the target population in public health setting oral cancer screening should be tobacco/alcohol users aged 35 years and above. A significantly reduced risk of advanced oral cancer was observed in a case control study of oral visual screening in the context of the Cuban national oral cancer screening programme [29].

Although a systematic review by the Cochrane collaboration [49, 50] and the US National Cancer Institute's PDQ cancer information summary (updated on 15 July 2011) on oral cancer screening [51] concluded that there was insufficient evidence to establish whether oral visual screening would result in a decrease in mortality from oral cancer, an expert panel of the American Dental Association (ADA) [52] recently concluded that community-based screening by means of visual and tactile examination may decrease oral cancer-specific mortality among people who use tobacco, alcohol or both, based on the evidence from the above described randomised trial [30, 31, 53].

The Cochrane collaboration concluded that the randomisation in the only randomised controlled trial [30] did not fulfil the CONSORT guidelines for cluster randomised trials; the study had small numbers of clusters; the study did not report data on costs, quality of life and harms of screening [49]. The authors of the randomised trial [30, 53, 54] responded explaining that CONSORT guidelines for cluster randomised trials did not exist in 1994 when the study was designed and that scientific and ethical approvals were obtained. Although the study fulfilled almost all CONSORT requirements, the authors explained that the smaller number of clusters was used in their study to avoid contamination between the intervention and control groups, given the high density of population; 835 of 3318 (26 %) screen-positive patients had healthy mucosa or benign lesions, constituting less than 1 % of eligible subjects; the low proportion of false-positive screening (and provision of treatment after biopsy confirmation) indicated low probability overtreatment in the study [30, 53]. The

authors emphasised the importance of recognising and implementing scientific evidence in a timely way, and the exclusion of evidence from randomised clinical trials done in developing countries from systematic reviews is a fact. The data on cost-effectiveness has now been published [30, 53].

The ADA expert panel recommended that clinicians should look for signs of PMDs or early-stage cancers while performing routine oral visual screening in all subjects, but particularly in those who use tobacco and/or alcohol. The panel also concluded that the life-saving benefits for subjects with treatable lesions identified through screening were more important than the potential harms incurred by those with benign or nonprogressive lesions [52].

10.10 National Oral Cancer Screening Programmes

The critical components of successful screening programmes are high coverage of target population with accurate, quality-assured screening tests and of screen-positive persons with diagnostic investigations, treatment and follow-up care, which are most cost-effectively met within organised screening programmes. Organised screening programmes for cancers such as cervical cancer have shown the greatest effect while using fewer resources than unorganised programmes.

No organised population-based oral cancer screening programme has yet been implemented in developed countries. There is no evidence that a significant proportion of the population above the age of 35 years or those at high risk, such as users of tobacco or alcohol, have received an opportunistic oral screening examination in many developed countries in the past 5 years. The cost- effectiveness of different oral cancer screening approaches such as organised invitational screen, as in the case of cervix cancer, opportunistic screening of the general population by general practitioners or dentists and opportunistic screening of high-risk population by general practitioners or dentists in the United Kingdom was simulated using a decision analysis model which showed that opportunistic screening of high-risk individuals by dentists may be cost effective, particularly for those aged between 40 and 60 years [55]. There have been a number of demonstration projects, involving several hundred people in developed countries, that have documented participation rates, screen positivity and disease detection rates, but these projects had no impact on the oral cancer outcomes at the regional or national level [56–58].

Despite the highest risk of oral cancer observed in South Asia and many studies in this region establishing the value of oral cancer screening, no national or regional screening programmes have been established in India, Pakistan or Sri Lanka. However, there are district-based programmes in Maharashtra and Kerala that provide oral visual screening through the primary care system.

A national oral cancer screening programme based on annual visual screening of individuals aged 15 years and above through existing primary dental care services is ongoing in Cuba since 1984 [27]. A descriptive evaluation of the programme in 1994 indicated that 12–26 % of the target population had been screened annually and only less than 30 % of screen-positive subjects reported for

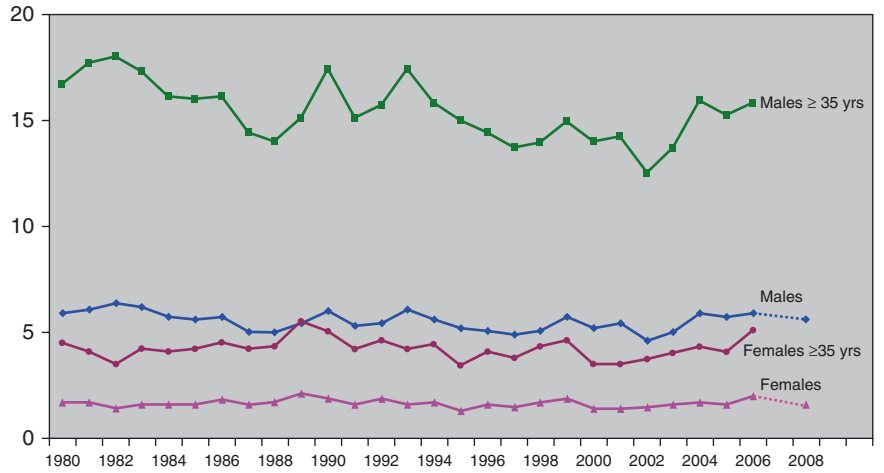

Fig. 10.5 Trends in oral cancer mortality rates in Cuba, 1980–2008 (World Health Organization. Mortality database. http://www.who.int/whosis/whosis. Accessed on 01/06/2010)

diagnostic investigations. The programme identified 16 % of 4412 oral cancer patients diagnosed in Cuba during 1984–1990; staging information was available for half of the cases, which showed that the proportion of stage 1 cases increased from 24 % in 1983 to 49 % in 1990 [31]. The programme was reorganised in 1996 with target age raised from 15 years to 35 years, screening intervals raised from 1 to 3 years, in order to improve coverage, and the referral system was revamped, but no further formal evaluation of the programme has been carried out. However, there has been no reduction in oral cancer mortality rates in Cuba over the last three decades (Fig. 10.5) [59] implying organisational and coverage deficiencies in the programme. The findings from the Cuban programme reinforce the fact that screening programmes, without efficient policies and organisation for invitation and coverage of appropriate target groups, referral systems and information flow, are bound to fail to deliver their objectives. An opportunistic oral cancer screening programme using visual inspection is ongoing in Taiwan, another region where oral cancer incidence is high (>20 per 100,000 people) and increasing, where 2 million people are betel nut/tobacco chewers and 6 million are smokers [60, 61]. The impact of visual screening on oral cancer incidence and mortality rates in Taiwan is not yet evident.

Conclusions

There is sufficient evidence that visual screening of the oral cavity can lead to early detection of OPMD and early oral cancers in asymptomatic people with tobacco use and/or alcohol drinking habits, leading to a reduction in oral cancer mortality in such high-risk subjects [30, 53]. It is a tool that all practitioners should use in routine clinical practice and should be made use of whenever a

Table 10.4 Future research directions in oral cancer early detection [62]

Evaluation of the performance and cost-effectiveness of adjunctive tests
Evaluation of the performance of salivary analysis as a screening tool
Determination of the natural history of well-categorised OPMDs in population-based longitudinal studies
Identification and evaluation potential predictive biomarkers
Determination of the long-term outcomes of treatment of OPMDs
Determination of the factors that predict and improve the participation of target populations in oral cancer screening
Evaluation of approaches to improve awareness and its impact on oral cancer detection
Determination of the reasons for the late presentation of oral cancers and delays in treatment
Documentation of the performance of ongoing national oral cancer screening programmes
Documentation of the trends in stage-specific incidence rates of oral cancer in population-based cancer registries and correlate the trends with ongoing opportunistic/organised oral cancer screening initiatives
Documentation of the harms and long sequelae of screening and quality of life issues
Evaluation of the utility of online and digital learning resources for training of healthcare providers

high-risk individual interacts with healthcare providers. The cost-effectiveness of oral cancer is improved by targeting those aged 35 years and above and who habitually use tobacco or alcohol [31, 55]. This implies that dentists and clinicians should be empowered to provide opportunistic oral visual screening during routine visits of high-risk individuals and should remain alert for early clinical signs of OPMD and oral cancer [52]. If population-based oral screening programmes are planned in high-incidence regions and countries, all components of the programme should be in place to ensure optimum coverage and performance, and its cost-effectiveness will be increased by targeting those aged 35 years and at high risk.

Important future oral cancer screening research priorities have been identified and these are summarised in Table 10.4 [62]. Addressing these issues will further improve our knowledge on oral cancer screening, will facilitate both opportunistic screening by clinicians and implementation of organised screening programmes and will reduce inequalities in oral cancer early detection and prevention, both within and between countries and populations [62].

It is not yet clear whether adjunctive screening tests such as visualisation aids and cytology are cost effective and improve oral cancer detection, and hence their routine use cannot be recommended. Large-scale longitudinal studies involving sufficient numbers of asymptomatic high-risk individuals in the general population with appropriate reference standard for final diagnosis are required to document their clinical utility in improving the performance of visual and tactile examinations. Population-based studies with long-term follow-up are required to further clarify malignant transformation rates of OPMD and the clinical utility of potentially promising biomarkers and the role of salivary analysis in the early detection and control of oral cancer.

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Prevention of Oral Cancer

11

Apurva Garg and Pankaj Chaturvedi

11.1 Introduction and Epidemiology

Oral cancer (ICD-10:C00-C08) is now a significant health issue in many parts of the world due to widespread use of tobacco, alcohol, and areca nut [1, 2]. According to GLOBOCON 2012, oral cancer is the 17th most common cancer in the world with about 300,373 new cases and 145,353 deaths in 2012 which is expected to rise to 406,022 and 197,811, respectively, by 2025 [3]. This cancer effects both the developed and developing countries but there is wide global difference in the incidence of this cancer (~20 fold) [2]. It is more common in South Asia (e.g., India, Sri Lanka, Pakistan, Taiwan), Latin America (e.g., Brazil, Uruguay, Puerto Rico and Cuba), Eastern Europe (e.g., Hungary, Slovenia, Slovakia), Western Europe (e.g., Germany, France), and Pacific regions (e.g., Papua New Guinea) [2]. It is the most common cancer among males and overall the 3rd most common cancer in India with age-standardized rate (ASR) of 7.2 but contributes highest to the number of new cases annually [3, 4].

The burden of oral cancer is high due to the costly treatment and increased morbidity and mortality [5]. The ASR of oral cancer is high in males of western countries due to increased consumption of alcohol and smoking [6]. The incidence rate is high in both sexes in the regions of South Asia and some African countries due to increased use of smokeless tobacco, areca nut along with smoking, and alcohol [6]. The mortality rate is relatively lower in developed countries due to easy accessibility of health services as compared to the developing countries which have sparse

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medical facilities and other socioeconomic issues [6]. Oral cancer is mainly a disease of the elderly with most cases between 50 and 70 years of age, but recently there is shift toward lower age of diagnosis. The mean age is between 51 and 55 years, reaching up to 64 years in Thailand. The male/female ratio varies from 1.45 in Japan to 10.5 in Taiwan but a reverse trend has been seen in Thailand (1.56) [5].

11.2 Pathogenesis and Risk Factors

World over the most common causes of oral cancer are tobacco (smoking and chewable), alcohol, and areca nut with other factors such as HPV, nutritional deficiencies, oral hygiene, genetic disorders, etc. [1]. The use of tobacco and alcohol consumption are estimated to be responsible for about 90 % of the oral cancer cases [7].

The population-attributable risks for alcohol consumption and smoking are about 74 % overall, 80 % for males and 61 % for females [6]. Oral malignancies are generally preceded by premalignant lesions like leukoplakia and erythroplakia. The progression to frank malignancy is a multistep and complex process which involves abnormalities of proto-oncogenes (ras, myc, erbB1), inactivation of tumor suppressor genes (p53, CDKN2A/p16^{INK4A}), etc. [8]. Inflammation and its mediators also play a very important part in oral cancer. These include prostaglandin pathways, inflammatory cytokines, reactive oxygen species, nitrogen species, nuclear factor kappa B, vascular endothelial growth factors, and microRNAs [9]. The role played by each of the important risk factor is described as follows.

11.2.1 Tobacco

Tobacco is the biggest risk factor for oral cancer in the world. It is any preparation of the leaves of plants belonging to the genus *Nicotiana* of nightshade family. Nicotine, the main psychoactive substance in tobacco, constitutes only 5 % of the total dry weight of the plant leaves [10]. It is used in two forms, smoking and smokeless, both of which are harmful (Fig. 11.1).

11.2.1.1 Smoking

Cigarette smoke has more than 60 carcinogens. The main carcinogens in tobacco are tobacco-specific nitrosamines (TSNA), e.g., N-nitrosonornicotine(NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone(NNK), polycyclic aromatic hydrocarbons (PAH), and other aromatic amines. The carcinogens exert their activity by forming DNA adducts [10].

Smoking: Tobacco is smoked in many forms like cigarette, bidi (tobacco handrolled in leaves of *Diospyros melanoxylon* or tendu), cigar, pipe, etc. [11]. The risk of oral cavity increases with duration, frequency, and the lifetime cumulative consumption of smoked tobacco. There is no safe level of smoking and even two cigarettes/day increases risk of oral cancer [11]. In a meta-analysis of 12 observational studies from 1961 to 2003 on tobacco smoking and cancer, the relative risk (RR) of

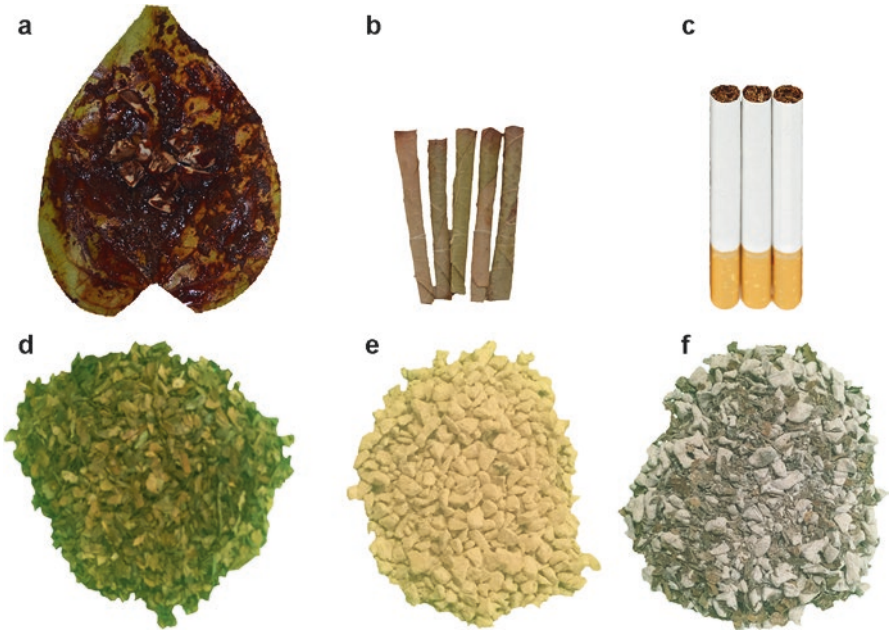

Fig. 11.1 Tobacco and areca nut products. (**a**) Pan (betel leaf with areca nut, tobacco, lime, and catechu). (**b**) Bidi (tobacco hand-rolled in *Diospyros melanoxylon* leaf). (**c**) Cigarette. (**d**) Packaged tobacco. (**e**) Pan masala (mixture of areca nut, lime, catechu, and added flavors). (**f**) Gutka (mixture of areca nut, tobacco, lime, catechu, and added flavors)

oral cancer among current smokers was 3.43 (95 % CI: 2.37–4.94) [12]. A case control study done in Brazil showed that the odds ratio (OR) for oral cancer with smoking ≤28 pack-years was 2.65 (95 % CI: 2.07–3.38) and ≥28 pack years was 7.43 (95 % CI: 5.94–9.30) [13]. In a meta-analysis comprising 12 case–control studies, the OR of oral cancer in bidi smokers as compared with nonsmokers was 3.1 (95 % CI: 2.0–5.0) [14]. Cigar smokers and pipe smokers had 7–10- and 2–3.5-fold increased risk of oral cancer as compared to nonsmokers [15].

11.2.1.2 Smokeless Tobacco

Globally it is used in many forms like gutka, khaini, kharra, mawa in India, maras in Turkey, toombak in Sudan, iq'mik in Alaska, and snuss in Sweden [16]. Smokeless tobacco has over 30 carcinogens and the important ones are similar to those found in smoking. The Global Adult Tobacco Survey (GATS) 2009 has showed that 25.9 % adults in India use smokeless tobacco [17]. Over half of the oral cancer cases in Indi and Sudan are due to smokeless tobacco but it is responsible for only 4 % cases in the USA. Data from the USA and Asia shows that the overall relative risk (ORR) for oral cancer associated with smokeless tobacco was 2.6 (95 % CI: 1.3–5.2), but it was not seen in studies from North Europe, ORR 1 (95 % CI: 0.7–1.3) [16]. A meta-analysis of 21 studies from Southern Asia had shown that the pooled OR for chewing tobacco and oral cancer was 4.7 (3.1–7.1) and for betel leaf (paan)

with tobacco was 7.1 (4.5–11.1) [18]. A case control study done in the USA on women using snuss showed an almost 50-fold increase in risk of developing oral cancer [19].

11.2.2 Alcohol

Alcohol is the 5th most common risk factor for global disease burden [20]. According to the WHO, it is consumed by over two billion people in the world and over 80 million have alcohol abuse disorders. It mainly contains ethanol which is a proven carcinogen with water and other minor components like nitrosamines, acrylamide, and oxidized polyphenols that are probable carcinogens to humans [10]. Seven to nineteen percentage cases of oral cancer are attributable to heavy drinking which is described as taking more than three drinks a day or five drinks on one occasion, once a week or daily drinking [10]. Ethanol is rapidly absorbed through stomach and small intestine from where it reaches the central nervous system and inhibits glutamate acting as a depressant. Habitual drinking in increased amounts leads to tolerance and development of dependence. Acetaldehyde which is a metabolite of alcohol is the main carcinogen and it leads to DNA mutation by forming adducts. There are different varieties of alcohol available such as wine, beer, whiskey, and vodka but case–control studies have found that their effect on oral cancer is independent of the beverage consumed [11]. According to a meta-analysis on drinking and oral cancer, the risk was similar in men and women. Compared to occasional or nondrinkers, the ORR for drinking was 2.55 (95 % CI: 2.15–3.02). The ORR for 1–2 drinks/day and >4 drinks/day was 1.36 (95 % CI: 1.20–1.54) and 5.40 (95 % CI: 4.49–6.50), respectively. This showed that there is no safe level of alcohol consumption. According to the type of beverage consumed, the pooled RR for never or occasional drinkers was 2.12 (95 % CI: 1.37–3.29), 2.43 (95 % CI: 1.78–2.98), and 2.30 (95 % CI: 1.78–2.98) for wine, beer, and spirits only, respectively [21]. A case–control study and two case series found that the floor of the mouth and the retromolar trigone were the most sensitive sites for cancer due to alcohol. In heavy drinkers the risk of developing cancer of the floor of the mouth and the retromolar trigone was 1.3–3.3 times and 2.2 times, respectively, as compared to tongue cancer [11]. According to a recent meta-analysis, the OR for oral cancer for ever drinkers was 4.21 (3.50–5.06), consuming ≤862 g-years was 1.68 (1.34–2.11), and consuming ≥862 g-years was 6.73 (5.35–7.91) [13]. According to another meta-analysis, the ORR for oral cancer in never or occasional drinkers was 1.17 (95 % CI: 1.01–1.35) and 4.64 (95 % CI: 3.78–5.70) for light and heavy drinking occasionally [22]. A recent meta-analysis on oral cancer and light drinking (≤1 drink/day) had shown that the RR for oral cancers was 1.17 (95 % CI: 1.06–1.29) [23].

11.2.3 Areca Nut

It is the fourth most used psychoactive substance in the world after nicotine, ethanol, and caffeine being used by over 10 % or 600 million people around the world [24]. Its use is mainly prevalent in South Asia, mainly India, Sri Lanka, Pakistan, Bangladesh,

Thailand, Vietnam, Taiwan, Papua New Guinea, etc. It is socially accepted in the Indian subcontinent and it is freely used by men, women, and even children in many forms like supari, pan masala, etc. (Fig. 11.1) [24]. Studies from Southeast Asia have shown that areca nut was being used by 25–50 % of the adults with up to 80 % use in rural areas [10]. It is mainly used with lime and catechu as a quid with or without betel leaf or tobacco. Its regular use leads to oral submucous fibrosis and various oral potentially malignant lesions (OPMLs) which often lead to cancer later on. The International Agency for Research on cancer (IARC) has declared it to be a group I carcinogen in 2003, being an independent factor for oral cancer [24]. The main metabolites of areca nut are arecoline, arecaidine, and guanidine which play a role in carcinogenesis. A number of nitrosamines and reactive oxygen species are also formed from the arecal alkaloids in the mouth, which play an important role in carcinogenesis, and among them the most carcinogenic is methylnitrosaminoproprionitrile (MNPN). The theory that areca nut is carcinogenic had come from Taiwan where 10 % of the population chews areca nut and more than 80 % of its preparations are tobacco-free [25]. The RR for oral cancer in Taiwanese who chew only areca nut was 58.4 (95 % CI: 7.6–447.6) [26]. Many case–control studies from Asia had shown that betel quid with or without tobacco was a bigger risk factor than smoking and alcohol in many areas. The OR for areca nut chewing was 2–11 and 2–3 times higher than alcohol and smoking, respectively [11]. In a study in South India, the OR of 4.2 and 16.4 was seen in men and women, respectively, who used areca nut without tobacco. The risk is substantially increased if the habit started before the age of 20 and frequency is ≥10 times/day. The risk increased with more duration and frequency and continued to be present for many years even after quitting [5]. In a South African study, 68 % of buccal mucosa and 84 % of tongue cancers were seen in areca nut users [27]. A meta-analysis of 15 studies had shown that the OR for developing oral cancer for usage of betel quid without tobacco was 2.82 (95 % CI: 2.35–3.4) which was presumably due to the areca nut present [28].

11.2.4 Combined Effects of Smoking, Tobacco Chewing, and Alcohol

Alcohol had synergistic effect on carcinogenesis when used in combination with tobacco [9]. A case–control study by Madani et al. showed that the OR for oral cancer due to combined effect of alcohol and smoking was 23.7 (95 % CI:12.6–44.6) and alcohol and bidi smoking was 19.6 (95 % CI: 4.6–83.5) [9]. A meta-analysis of observational Southeast Asian observational studies showed that the pooled OR for drinking–smoking–chewing was 40.1 (95 % CI: 35.1–45.8). Among these subjects, the individual effects of drinking, smoking, and chewing were 3.1 %, 6.7 %, and 17.7 %, respectively, while the rest, 72.6 %, was due to the interaction effect [29]. A nested case–control study from Southern India studying the interaction between smoking, chewing, and drinking in oral cancer showed that the OR for smoking–alcohol, chewing–alcohol, smoking–chewing, and smoking–drinking–chewing was 2.6 (95 % CI: 1.4–5.0), 6.4 (95 % CI: 2.8–14.6), 5.5 (95 % CI: 2.6–11.4), and 4.8 (95 % CI: 2.5–9.3), respectively, in males [30].

11.2.5 Human Papillomavirus (HPV)

HPV is one of the most powerful carcinogens and is mainly responsible for cervical, anogenital, and oropharyngeal cancer (OPC) [31]. It is responsible for about 3 % cases (but not exclusively) of oral cancer which is generally attributed to sexual behavior, and the most common subtypes implicated are 16 and 18 [10, 11]. According to Kreimer et al., the prevalence of HPV in oral cancer in Asia, Europe, and North America was 33 % (95 % CI: 30.3–35.8), 16 % (95 % CI: 13.4–18.8), and 16.1 % (95 % CI: 13.2–19.4), respectively, with the overall global prevalence being 23.5 % (95 % CI: 21.9–25.1 %) [31]. The available data did not show any interaction between tobacco and HPV in non-oropharyngeal sites contrary to OPC [31]. A meta-analysis on association between hpv-16 and oral cancer showed OR 2 (95 % CI: 1.2–3.4) [11]. HPV-DNA was most common in the cancers of the floor of the mouth and the tongue. It was found in 81.1 % of the cancers of the oral cavity associated with HPV and OR of oral cancer for HPV-16 was 2.8 (95 % CI: 1.2–6.6) [11]. Herrero et al. showed that the OR associated with HPV16 E6 and E7 seropositivity for oral cancer was 3.4 (95 % CI: 1.6–7.3) [31]. The role of HPV vaccination in prevention of oral cancer was not clear, but the increase in the prevalence of HPV+ OPC cases favors vaccination against HPV in both males and females. Both the univalent and quadrivalent variants of available HPV vaccines were effective against the oral transmission. A randomized trial conducted in young Costa Rican women showed that the efficacy of the quadrivalent vaccine was better against oral as compared to cervical HPV infection (efficacy 93.3 % (95 % CI: 62.5–99.7 %) vs 72 % (95 % CI; 63–79.1 %)) [32].

11.2.6 Oral Hygiene

Apart from the major risk factors, tobacco and alcohol, oral hygiene and infections are also associated with increased risk of oral cancer [33, 34]. The exact mechanism of carcinogenesis due to these factors is not clearly understood, but it is suggested that infections may trigger cell proliferation, interfere with cellular signaling, inhibit apoptosis, and thus promote tumorigenesis. A meta-analysis of 18 case–control studies showed that as compared to highest brushing frequency, the lowest brushing frequency had OR of 2.08 (95 % CI: 1.65–2.62) for oral cancer [33]. Periodontal disease had shown to increase the risk for head and neck cancer with OR 4.36 (95 % CI: 6.01–93.16) [34].

11.2.7 Trauma

Repeated irritation from dental factors has been proposed as emerging risk factor for oral cancer. Oral cavity cancers occur mainly at sites of potential dental trauma like the lateral border of the tongue (sharp tooth) and gingiva (ill-fitting dentures) especially in nonsmokers without other risk factors [35, 36]. Repeated mechanical irritation may be caused by an intraoral injury agent like defective teeth

(malpositioned or with sharp or rough surfaces), ill-fitting dentures, and parafunctional habits (e.g., tongue interposition or thrusting, oral mucosa sucking or biting). These factors could generate lesions or intensify previous oral diseases, thus promoting oral cancer [35]. A case–control study had shown that chronic ulcers due to lose dentures had adjusted OR of 4.58 (95 % CI: 1.52–13.76) [36].

11.2.8 Genetic Disorders and Family History of Cancer

Studies have shown that genetic susceptibility and family history also play an important role in oral cancer. Genetic disorders lead to inefficient metabolism of carcinogens/pro-carcinogens, increased mutagen sensitivity, or inability to repair DNA damage, thus predisposing an individual to various cancers [37]. *P53* mutations are more common in Western populations, whereas RAS oncogene mutations are more common in India and South-Asian populations [38]. Mutations in metabolic enzymes cytochrome450, glutathione S-transferase (GST), N-acetyl transferase (NAT), and alcohol dehydrogenase (ADH) predispose to oral cancer [38]. A large case–control and cohort study showed that risk of oral cancer was multiplied by 1.2–3.8 times in subjects who had a first-degree relative with history of head and neck cancer [11].

11.3 Prevention of Oral Cancer

Prevention is the most cost-effective strategy for long-term control of cancer [6]. There are four levels of prevention, corresponding to the different phases in the development of a disease: primordial, primary, secondary, and tertiary. Majority of oral cancer are due to the use of tobacco, alcohol, and areca nut and avoiding these substances can bring down the oral cancer burden substantially.

National cancer programs can help the governments to utilize the available resources for the benefit of the people [6]. The 58th World Health Assembly Resolution on Cancer prevention and control (WHA 58.22) requires the member states to implement comprehensive cancer control programs suited to their socioeconomic needs in collaboration with the WHO and devise strategies for detection, diagnosis, early treatment with proper rehabilitation, and palliative services [6]. Modifiable risk factors, premalignant lesions, long latent period, and full treatment of early cases are the basis of primary and secondary prevention of this disease [1]. Creating awareness, screening programs, and strict implementation of laws for tobacco and alcohol control are the best ways for preventing oral cancer morbidity and mortality.

11.3.1 Primordial Prevention

The aim of primordial prevention is to prevent the emergence and establishment of the social, cultural, and economic patterns of living that contribute to an enhanced

risk of disease. Primordial prevention of oral cancer should incorporate a national policy to discourage use of tobacco, alcohol, and areca nut [39]. Children are the best suitable population group for this type of prevention. They can be made aware about the harmful effects of tobacco and alcohol so that they are discouraged from taking up these habits in future [40].

11.3.2 Primary Prevention

The aim of primary prevention is to limit the incidence of disease by controlling causes and risk factors [39]. After 5 years of quitting smoking, the risk of oral cancer is decreased by up to 50 % and it approaches the level of nonsmokers after 20 years [1, 2]. The risk of oral cancer with alcohol is also reversed after 20 years of abstinence [41]. In South Asia, the method to decrease the incidence by reducing the habits of betel quid chewing has shown results where a reduction in incidence of oral cancer has been noted [1]. Heavy users need the services of primary caregivers and specialist cessation clinics as relapse rates after quitting are high [1, 2]. Various studies conducted in rural India had shown the effectiveness of primary prevention where various communication methods like radio, newspapers, films, and personal communications had led 15 % to quit tobacco use and many others to decrease the use substantially [42].

A case–control study comprising house to house survey was done in three parts of rural India involving 36,471 tobacco users. The individuals were interviewed about their tobacco habits, examined for pre-cancerous lesion and given personal advice as well as by mass media to give up their tobacco habits. In two out of three areas, substantially more people decreased the frequency of use and gave up their tobacco habit as compared to the control group, while the remaining third area showed slightly higher proportion of people quitting this habit with no difference in reduction of frequency of use. The 5-year incidence rate among men and women in one district dropped from 47.8 and 33 to 11.4 and 5.8, respectively, in the intervention group, while in the another district it dropped from 260.8 and 489.5 to 59.8 and 28.5 in men and women, respectively [43]. Just extra 2–3 min taken by clinicians had shown the change in attitude, knowledge, and behavior of people toward tobacco use [42]. Government legislation and enforcement to ban gutka (a mixture of tobacco, areca nut, and other flavorings) has shown to be effective in reducing use and sale.

A cross-sectional study in Indian state of Maharashtra involving 68 users and five tobacco vendors showed that after the ban on gutka 23.53 users quit this habit with 55.88 % showed reduced consumption. Both the users and vendors were in favor of the ban and 45.6 % users got the information about the ban from electronic media [44]. In South Africa an increase in excise tax on cigarettes leading to increase in real price of cigarettes by 10 % lead to 5–7 % decreased consumption of cigarettes, if other factors were constant [44]. The reduction in use was more pronounced among the poor and the government revenue increased in spite of decreased cigarette consumption [45].

Voice of Tobacco Victims (VOTV) is a nonprofit organization working in India comprising cancer survivors and motivated oncologists all over the country. They are doing voluntary advocacy with the policy makers and battling the powerful tobacco industry. This campaign has played the chief role in the pan-India gutka ban, increased taxes on tobacco products, and decline in volume sale of chewable tobacco and cigarettes by 26 % and 3 %, respectively [46]. To draw the world's attention on effective care and control of head and neck squamous cell cancer (HNSCC), the International Federation of Head and Neck Oncologic Societies (IFHNOS) declared 27 July as World Head and Neck Cancer Day (WHNCD) on the occasion of its 5th World Congress in New York on 27 July 2014 [47].

11.3.3 Secondary Prevention

It includes early detection of cancer through screening programs in individuals who are asymptomatic or at risk as well as prevention of malignant transformation of OPML [41]. Its aim is to reduce the prevalence of a disease and is directed at the period between onset of disease and the normal time of diagnosis. It can be only applied to diseases which have an easily identifiable early period in the natural history when it can be identified and treated so as to prevent further progression [39]. Oral cancer is an ideal model for screening as it meets all the criteria of principles of screening (Table 11.1) [48]. Treatment of oral cancer at early stages achieves increased survival rates and decreased morbidity, but unfortunately many patients present with advance disease [1].

A cluster randomized trial involving 191,873 subjects in Southern India showed that after 15 years of follow-up and four rounds of screening, there was a statistically significant decrease in incidence and mortality of oral cancer by 38 % and 81 %, respectively [49]. A Cochrane review published in 2013 had recommended screening of high-risk individuals with tobacco and/or alcohol use [50]. This is also supported by recommendations by various health agencies across the globe like the American Cancer Society, Canadian Task Force, and National Health Services UK [51–53]. This has shown to be the most effective strategy and will lead to improvement of survival and stage shift across whole populations [50]. The primary care health workers, general practitioners, and dentists have an important role in screening of such individuals. The gold standard for screening is visual examination which has sensitivity and specificity up to 98 % [51]. High-risk individuals should do regular mouth self-examination (MSE) in front of the mirror under good illumination

Table 11.1 Principles of screening [48]

1. Important to public health
2. Reasonable balance between the cost of screening, including its consequences in follow-up and treatment and effectiveness of results
3. The natural history must be well understood
4. Available information on the validity of screening tests and effectiveness of early treatment
5. Ability to reach the population at risk with proffered tests

Table 11.2 Various diagnostic modalities for screening of oral cancer [54]

Lugol's iodine
Toluidine blue
Oral brush biopsy (OralCDx)
Tissue fluorescence imaging (VELscope)
Tissue fluorescence spectroscopy
Light-based detection systems
Chemiluminescence (ViziLite Plus; Microlux/DL, Orascoptic-DK)
Laser capture microdissection
DNA analysis
Biomarkers
Excision biopsy and histopathology

and look for any suspicious white or red patches, ulcers, or swellings and report to physicians/dentists if any of the above features are seen [51].

A spectrum of diagnostic modalities is available for screening of oral cancer, ranging from the traditional methods like Lugol's iodine and methylene blue to the latest methods using DNA analysis, laser capture microdissection (Table 11.2) [54]. These are not described in detail as they will be covered in other sections of the book. Surgical intervention has also been tried as a method of treatment of OPML, but a recent meta-analysis showed that the risk of transformation is decreased but not eliminated completely [55]. To reduce the risk of transformation, the modifiable risk factors should be eliminated and the subjects should be under regular follow-up. The chance of transformation is highest in the first two years and 1 %/year thereafter and suspicious lesions should be biopsied by experienced clinicians [55].

11.3.4 Tertiary Prevention

Effective initial management of oral cancer with improved survival has lead to the emergence of second primary tumors (SPTs) [1]. Tertiary prevention aims to prevent or detect SPTs at the earliest [41]. The National Cancer Care Network (NCCN) suggested that cured patients need to be under regular follow-up for early detection of complications of treatment, recurrences, and SPT in the lung [41]. There is higher chance of recurrences and SPTs in individuals who continue to smoke after treatment or were treated with radiotherapy alone [1]. A study done in Southern England estimated that after 20 years of the first head and neck malignancy, males and females had approximately 30 % and 20 % chance of developing a SPT, respectively [1].

11.4 Chemoprevention of Oral Cancer

It involves the use of natural products or synthetic drugs (Table 11.3) that can reverse or arrest malignant transformation of OPML to address the issues related with field cancerization [41]. It is based on several mechanisms such as inhibition

Table 11.3 Various agents for chemoprevention [5, 7, 41, 55]

1. Retinoids
2. Black tea and green tea polyphenols
3. Cyclooxygenase inhibitors
4. EGFR inhibitors
5. Thiazolidinediones
6. Vitamin C
7. Curcumin
8. Bowman–Birk inhibitor concentrate
9. Photodynamic therapy with aminolevulinic acid
10. Blackberry gel
12. Natural products like lycopene, withaferin A, essential oils, berberine, resveratrol, etc.

of DNA adduct formation; scavenging of reactive oxygen species; inhibition of JAK/PKCd/STAT1 signaling pathway; neutralization of carcinogens; regulation of p53, p21, p57, and Bax; etc. [10, 41, 56].

Retinoids are the most extensively studied class of drugs for chemoprevention [41, 56]. Their main sources are carotenes from plants and retinyl esters derived from animals. They regulate the growth and squamous differentiation by restoring the expression of retinoic acid receptors [55]. A double-blind placebo-controlled trial on leukoplakic lesions using high-dose 13-cis retinoic acid reported that there was 67 % and 54 % improvement in clinical response and histological improvement. This was however associated with hypertriglyceridemia, severe mucocutaneous reactions, and frequent relapses after cessation of treatment [41]. Similarly use of vitamin A and/or beta-carotene for leukoplakia also showed relapses after showing clinical improvements [55]. A randomized control trial done to study the effect of low-dose beta-carotene and vitamin C supplements on leukoplakia failed to show any effect on clinical remission or protection against malignant transformation [57]. A randomized control trial using the same drug for tertiary prevention in treated cases of stage I–IV head and neck squamous cancer lowered incidence of SPT but further trials failed to show this effect. Separate randomized controlled trials studying the chemopreventive effects of cyclooxygenase-2 (COX-2) inhibitors celecoxib and ketorolac did not demonstrate any difference between the intervention and non-intervention arms [41]. A randomized control trial using peroxisome proliferator-activated receptor gamma (PPARγ) agonists thiazolidinedione and pioglitazone showed 68 % clinical/histological response as compared to 0 % in placebo. Further phase IIb studies using pioglitazones were underway and results are awaited [41]. TP53-targeted agent ONYX-015 is an attenuated adenovirus which targets cells with dysfunctional p53 pathways. It had shown some promise when used as a mouthwash or injected in the dysplastic mucosa but the responses were short-lived and did not address the remaining mucosa at risk, respectively [41].

Various natural products like green tea extract, curcumin, blackberries, lycopene, and resveratrol have also been tried as chemopreventive agents [56]. A recent meta-analysis on effect of tea consumption and risk of oral cancer showed that the relative risk (RR) of oral cancer for the highest versus lowest category of tea consumption was 0.853 (95 % CI: 0.779–0.934) [58]. The consumption of green tea showed RR

of 0.798 (95 % CI: 0.673–0.947) which was significant but not for black tea (RR 0.953 (95 % CI: 0.792–1.146)) [58]. Another trial using green tea extract showed clinical/histological response in some patients but did not prevent malignant transformation [55]. EGFR inhibitor erlotinib is being studied in many trials with or without celecoxib across the world as a chemopreventive agent [41, 55]. Recently published results of a trial using both drugs were encouraging and showed 71 % response rate in carcinoma in situ and pathological dysplasia. The results of further trials are eagerly awaited [41, 55].

Conclusion

Oral cancer is an important health problem in many parts of the world especially South Asia. Almost half of these patients die within a year of diagnosis due to late presentation. The oral cancer burden can be reduced dramatically by avoiding tobacco, alcohol, and betel quid as they are responsible for more than 90 % of the cases. It is one of the best models of screening, and lot of morbidity and mortality can be reduced if the cases are detected early. The primary health professionals have an important role to play in screening and also sensitizing the users about the harmful effects of the risk factors and eventually quitting them. The role of chemoprevention is still investigational due to inconsistent results and lack of large randomized control trials. National cancer control programs involving screening, diagnosis, treatment, rehabilitation, and palliation are vital in control of this disease. Government policies on restricting production and sale, ban on advertising, raising taxes, and strict implementation of laws are highly effective strategies for control of tobacco and alcohol and it has been proven in various studies across the world.

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Biologic Basis of Personalized Therapy in Head and Neck Squamous Cell Carcinoma

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Head and neck squamous cell carcinoma (HNSCC) is the 7th most common cancer worldwide, with more than 500,000 new cases each year [1]. HNSCCs are associated with a 5-year overall survival of approximately 50 % which has remained largely unchanged [2]. Cetuximab is the only FDA-approved targeted therapy for HNSCC and there are no clinically used biomarkers for HNSCC. Personalized therapy, treatment management customized to individual patient profile, is now being investigated with greater emphasis on the molecular profile of the patients, in addition to their clinical and pathological status. With the stagnant 5-year overall survival, this shift toward molecular integration is definitely warranted. Molecular profiling has the ability to categorize the patients based on effective treatment modality, treatment response and susceptibility to develop metastasis, thereby delineating the prognosis more accurately. Response assessment further enables selection of drugs based on the status of the targeted pathways, hence ensuring better treatment outcome. Nevertheless, the success of this approach is dependent on the selection of appropriate biomarkers. Differences in the biology of cancers in terms of characteristics such as site, tissue of origin, and etiology make it mandatory

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to catalog the changes that are specific to each cancer as a step toward the personalized medicine approach. The advent of high-throughput techniques has enabled a global view of the molecular changes that occur at every level and hence are arguably the best option to understand and identify the probable clinically relevant and targetable pathways in HNSCC.

12.1 High-Throughput Data

Global profiling facilitates a deeper understanding of the processes that render individual patients different in terms of susceptibility to progression, response to treatment, and development of metastasis, the basis behind the concept of personalized medicine. The molecular understanding of oral carcinogenesis is further challenged by factors such as prevalence of risk habits, late stage of presentation, and influence of HPV. Genomic, transcriptomic, and proteomic profiling studies have been carried out in oral cancer; cataloging of the repertoire of markers can provide insights into the possible biomarkers that can be applied in personalized medicine.

12.1.1 DNA

Advances in high-throughput profiling technologies such as the advent of next-generation sequencing have revolutionized the field of global profiling of genome-based changes, both in terms of wealth of information obtained and the kinds of abnormalities detected, ranging from single-nucleotide variations to large structural rearrangements. The primary challenge, however, has been to extract clinically relevant and applicable information. It is of utmost importance to discern the practical application of information obtained to benefit patients.

12.1.1.1 Somatic Mutations

Exome sequencing of squamous cell carcinomas of head and neck cancers have pointed out several candidate genes that are mutated across the cohorts of patients analyzed. A prime candidate that has arisen in multiple studies is the NOTCH1 gene and the associated pathways; 15–50 % of the HNSCC patients and 60 % of patients with premalignant lesions showed mutations in the gene [3–7]. The members of the Notch1/p63 axis which includes FBXW7, known for its role in the differentiation of squamous epithelium, were also found to be extensively mutated in patients with HNSCC [5, 6], indicating the significance of the pathway. In-depth analysis of the type of mutations observed indicates that most of the patients harbored missense or nonsense mutations with the Notch ligands being rarely mutated. The increased incidence of the mutations in the premalignant lesions indicated that the pathway/gene might be a possible driver of oral carcinogenesis [3, 8]. A correlation with clinical outcome indicated that patients with Notch1 mutation had shorter overall and disease-free survival with multivariate analysis revealing Notch1 mutation to be an independent prognostic factor in the patient population [8]. A deeper

understanding of these mutations and the hotspots specific to head and neck cancers will probably be essential to evaluate their clinical utility and devise novel targeting strategies.

The other genes that came into focus for their extensive genomic alterations in head and neck cancer, both in patients and cell lines, are TP53 and PI3KCA [4–7, 9, 10]. Along with CDKN2A, these genes were mutated in more than 30 % of the patients with the disease. The PI3K pathway is identified to be the most frequently mutated and potentially targetable pathway (>30 %). A further analysis revealed that these mutations were primarily observed in patients with advanced stages, indicating their association with progression of disease. *In vivo* studies also indicated the resistance of these tumors to mTOR/PI3K inhibitors [11]. The Cancer Genome Atlas profiling the genomic landscape of somatic alterations showed that while helical domain mutations of PI3KCA are associated with HPV-positive tumors, smokers demonstrated loss of function TP53 mutations and CDKN2A inactivation, indicating a possible correlation with etiology of the disease [7].

12.1.1.2 CNVs

Copy number variations (CNVs) in the genetic makeup of the patients with oral cancer have been documented; DNA amplifications were predominantly observed in the regions 8q22.2 and 8q24.3 [12, 13]. An integrated analysis of CNVs with transcript-level differences indicates that the MYC regulatory network showed the highest number of CNV-associated transcripts. More than 25 % of the transcripts identified are associated with second primary tumors, relapse, and survival. In addition, copy number gains in other areas such as 3p, 6p, 11q, 16p, and 17q and losses in 2q, 3p, 4q, 8p, 9p, 11q, and 18q were associated with head and neck cancer. The major subset of genes which showed losses included NFkB1, IL2, TUSC3, CASP1, ERBB2, FADD, CTTN, and GATA4, while gains were observed in MYC, VEGF CDKN2D, BRCA1, CCND1, and FGF3 [13, 14]. CNVs observed in GSTM1, the gene involved in detoxifying pathways, have been shown to be better predictors of recurrence and survival [15].

Tracing the CNVs in premalignant lesions and carcinomas developing in the same site showed copy number gains in 1p in 80 % of the cases with the other frequently observed amplifications being in 11q, 9q, 21q, and 6p along with 22q, 10q, and 7p. In the 11q region, TAOS1 and EMS1 showed progressive increase in amplification during oral carcinogenic progression from normal mucosa to premalignancy to malignant lesions [16]. Losses in copy numbers were observed mainly in 16p, 9q, 17q and 80. These CNVs mapped in both the premalignant lesions and the corresponding oral squamous cell carcinoma (OSCC) were the loci of the genes PARP1, BTBD7, and RAB1A, suggested to be indicators of progressive disease [17]. Studies in EGFR and p53 also indicated common copy number variations in leukoplakia and cancers [18, 19].

12.1.1.3 Indels

Insertions/deletions (indels), the mutational change that refers to a combination of insertion and deletion of nucleotides in the chromosome, in cancer-associated pathways, are relevant disease markers. The insertion/deletion polymorphism in the

ACE gene (angiotensin 1-converting enzyme) was high in OSCC patients, with the risk being high in homozygotes [20]. The other polymorphism associated with risk of oral cancer was in the NFkB gene (−94ins/del) [21].

12.1.1.4 Tumor Heterogeneity

Tumor heterogeneity is one of the prime challenges that need to be addressed in order to successfully manage patient-specific issues associated with cancer diagnosis and therapy management. The significant genomic alterations and instability observed in head and neck cancers further signify the possibility of extensive molecular heterogeneity in the tumors. MATH (mutant-allele tumor heterogeneity), representing the ratio of the width to the center of the mutant allele fraction distribution among the tumor-specific mutated loci, was defined as a measure of heterogeneity in patients with head and neck cancer [22]. Intra-tumor heterogeneity, as represented by this statistic, was higher in patients with poor outcome, mutant TP53, negative HPV, and high exposure to risk habits. Heterogeneity, quantified in this manner, provided a clinically applicable biomarker that could be correlated with prognosis and treatment outcome [22, 23].

Comparative genomic hybridization (CGH) studies indicated that the highest rate of heterogeneity was observed between the primary tumor and its corresponding metastasis with the rates being highest in the oral cavity (49.2 %) as compared to matched sites of metastasis or distinct sites of primary tumor [24]. These studies indicated that heterogeneity is a function of the genetic background of the patient and etiology of the disease along with the stage of tumor progression, further signifying its relevance and the need to adopt this parameter in personalized medicine.

12.1.1.5 HPV

Human papillomavirus (HPV), a causative factor in cervical cancer, is now considered a significant etiological concern for head and neck cancer. The association with oropharyngeal cancer has been well established in previous studies [25]. The viral mode of action is through inactivation of p53 and the RB pathways. Patients with HPV-associated carcinogenesis have fewer genetic alterations and better treatment response and survival rates [4, 6, 26]. Next-generation sequencing (NGS) studies in oropharyngeal cancers have identified varying mutation profiles in HPV+ and HPV− patients; 100 % of patients in the negative cohort were positive for p53 with CDKN2A deletion/CCND1 amplification, indicating an entirely different mode of carcinogenesis in the two cohorts [27, 28]. The SNPs identified were reported mainly in the tumor suppressor genes and zinc finger proteins in the HPV (−) patients while the tyrosine kinase receptors had a greater number of sequence variations in HPV (+) patients [29, 30]. Integration of the virus into the genome is also reported to have profound impact leading to extensive copy number variations, chromosomal rearrangements, methylation patterns, and transcript-level differences, further providing insights into the mechanisms involved in viral-induced carcinogenesis [31]. In the background of these extensive differences in the molecular profile of the two groups, the use of HPV to categorize the patients prior to treatment decision making will be an important clinical parameter.

12.1.2 Epigenetic Modifications

Aberrant epigenetic modifications play an important role in the process of carcinogenesis and tumor progression, and several high-throughput methylation studies have identified the methylation hotspots in head and neck cancers. Genome-wide screen using restriction landmark genomic scanning identified a batch of genes that were commonly methylated (Septin 9, SLCFA8, FUSSEL18, EBF3, and IRX1) in up to 67 % of the patients with HNSCC with HPV independent etiology [32, 33]. These genes were associated with the TGFβ signaling pathway that is usually disrupted in HNSCC, and their decreased expression leads to reduced apoptosis accompanied with increased proliferation and reduced differentiation [34]. In tongue cancer, global screening using methylated DNA immunoprecipitation and microarray revealed a subset of genes that were hypermethylated (FBLN1 and ITIH5) or hypomethylated (RUNX3) as compared to the tumor-adjacent mucosa. *In vitro* studies have also identified extensive HOX gene hypermethylation in oral cancers (>50 %) [35].

12.1.3 Transcript Level

12.1.3.1 Array-Based Profiling

Alterations at the genomic level owing to mutations or large structural rearrangements ultimately lead to extensive change in the expression profile of transcripts. Changes at the transcript levels are thus indicative of the clinical relevance of the genomic changes and ratify their significance in the carcinogenic process. Global profiling of transcriptomic changes by microarrays has been suggested to be indicative of susceptibility of premalignant lesions to progression, metastasis, and treatment outcome.

A review of a majority of the microarrays carried out in oral premalignant lesions indicated 31 genes that were common across at least two studies and hence can be investigated with high confidence for their role as predictive biomarkers [36]. Characterization of leukoplakia of various dysplastic stages identified an 11-gene signature that can distinguish leukoplakia from oral cancers and 7 genes that can differentiate the lesions with different grades of dysplasia [37]. Two-gene signatures (LAMC2 and COL4A1; COL1A1 and PADI1) were also able to distinguish dysplasia from normal tissue [38]. The molecular profile of lesions susceptible to progression was further characterized by persistent pro-inflammatory conditions with repression of key enzymes in the arachidonic pathway (prostaglandin D2 synthase-PTGS). Increased expression of genes involved in invasion such as ISG15, PSOR1 and CSPG2 were also observed in the oral premalignant lesions and carcinoma [39, 40]. Profiling of lichen planus (LP), a potentially precancerous lesion, and subsequent validation reported a strong correlation of Topo IIa with dysplastic changes in the samples with a high predictive value to detect LP lesions prone to malignant transformation [41]. Assessment of oral cancer signatures in comparison to the adjacent normal mucosa has led to the identification of a large subset of deregulated

genes in the tumor. The major pathways identified were those involved in cell communication, integrin-mediated cell adhesion [42]. A 25-gene predictor was reported to classify oral cancer specimen with 96 % accuracy and 87 % specificity for oral tumor alone [43].

Progression of head and neck cancer leading to metastasis, regional to lymph nodes or distant, is one of the major factor affecting survival of patients with head and neck cancer. Profiling of patients with susceptibility toward developing metastasis will enable accurate management, leading to improved prognosis. Microarray profiling and subsequent validation revealed upregulation of CCL19, CD2, EGR2, FUCA1, RGS1, SELL, MMPs, uPA, TNC, integrin-α and downregulation of IGFBP6 and KLK8 to be associated with nodal metastasis [44] with a separate study identifying MMP1 and integrin-α as the most significant predictors [45]. An 8-gene prediction model (DCTD, IL-15, THBD, GSDML, SH3GL3, PTHLH, RP5-1022P6, and C9orf46) for lymphatic metastasis was also proposed for oral cancer, which can be clinically applied, subject to further validation [46]. In tongue cancer, GLUT3 and HSAL2 demonstrated correlation with depth of invasion, advanced T stage, and disease-free and overall survival [47], while markers such as BAG4, PAX3, and CCN1 were markers of progression [48]. The markers that correlated with metastatic potential included molecules of cell mobility (SNTA1), adhesion, and ECM proteins (ADAMTS2 and Cathepsin O) [48].

Resistance to treatment is accomplished by deregulation of multiple pathways involving DNA repair, detoxification, cell cycle, and apoptosis. Understanding these molecular players is probably the biggest challenge and one of maximum utility in disease management in oral cancer. An informed decision based on the molecular profile that can determine the patient response will be of extreme benefit in terms of improving outcome and survival. Although numerous studies have been carried out on cell lines listing the possible pathways that determine resistance/response to different therapies, studies on patient cohorts are comparatively few. In tongue cancer, profiling of patients who responded to treatment as compared to those who did not identified a panel of genes (COL5A1, HBB, IGLA, and TSC) as associated with treatment response [49]. Resistance to chemoradiotherapy (platinum derivatives) in advanced HNSCC patients was reported to be conferred by low expression of caspase-8 and an overexpression of MDR-3 and p-Gp proteins. Tumors with poor outcome and short disease-free survival also showed an overexpression of antiapoptotic factors (p-53, BCL-2, BCL-x) [50].

12.1.3.2 RNA Sequencing

Advancements in NGS-based sequencing have further broadened the scope of information from transcriptome profiling. RNA sequencing of oral cancer samples when compared to the adjacent normal mucosa identified a subset of genes (ANKRA2, GTF2H5, STOML1, NUP37, PPP1R26, and TAF1L) that distinguished the tumor [51]. Similar studies have also shown that P53 signaling and transcripts of actin-mediated cell contraction were deregulated in buccal mucosal cancers with downregulation of transcripts such as MYL1, ACTA1, TCAP, and desmin [52].

Massively parallel sequencing has widened the scope of transcriptomic events that can be cataloged with the detection of fusion transcript, splice variants, and

isoforms in head and neck cancer. High-throughput sequencing in combination with a screen for splice variants in buccal mucosal cancers identified 11 novel splice junctions mostly at the 5′splice site along with a novel IgG pseudo-gene and a fusion transcript of MEMO1 and RPS9. These splice variants were identified in the adjacent normal tissue, indicating their involvement in cancer development and progression [53]. A global study profiling fusion transcripts in all cancers showed that at least 1 % of head and neck cancers had potentially druggable kinase fusions involving ALK, ROS, RET, NTRK, and FGFR genes [54]. Extensive studies and clinical trials are essential to further explore the fusion transcripts that may be prevalent in head and neck cancers and to establish their clinical utility in the disease.

12.1.4 miRNA

MicroRNA, a significant component of the regulatory network, has also been studied to evaluate their clinical utility as biomarkers. In head and neck cancer, a subset of 12 miRNAs has been identified that are independent prognosticators of recurrence-free survival and 4 miRNAs that correlate with cancer-specific survival [55]. Cataloging of the aberrant miRNA expression in oral cancer patients as compared to healthy volunteers indicated a 21-miRNA panel that was significantly differentially expressed [56]. This panel which includes miR-494, miR-3651, and miR-186 may serve toward development of predictive marker panel. A 13-miRNA subset was further identified to be associated with progression and metastasis in oral cancer, the downregulation of miR-155, miR-146a, and let-7i characterizing metastatic tumors [57]. In addition, aberrant methylation of miR-375, miR-200a, and miR-200c-141 could distinguish oral cancer patients from oral rinses as well as saliva, suggesting a potential clinical application [58].

12.1.5 Proteomic Profiling

Proteomic profiling using advanced technologies, such as mass spectrometry, iTRAQ-LC-MS/MS, and MALDI-TOF, has resulted in a documentation of the global changes that characterize head and neck carcinogenic progression. Assessment of global proteomic changes during dysplastic progression identified SFN, YWAZ, and hnRNPK to be predictive of oral premalignant lesions [59]. Further correlation of these markers to prognosis indicated increased expression of YWHAZ and SFN to be correlated with reduced disease-free survival (13 vs 38 months), emphasizing their role as adverse prognosticators [60]. Laser capture microdissection (LCM), in combination with LC-MS/MS, identified keratin 13 as differentially expressed (downregulated) in the tumor samples, while placental growth factor (PIGF) was detected only in the tumor samples [61]. Proteomic analysis has also implicated other pathways such as interferon signaling and molecules such as PRDX4 and P4HA2 [62, 63]. Marker panels specific to saliva, plasma, and serum in patients with premalignant disease and oral cancer have extreme

significance as early diagnostic markers, such as DUSP1, IL8, IL6, S100P, haptoglobulins, and ribosomal s6 kinase [64–66]. These markers are yet to be evaluated for their utility in point of care assay systems.

"Omics" analysis in head and neck cancer and subsequent correlation with clinical characteristics has listed major pathways that can be investigated for their relevance as predictive biomarkers and targetable molecules. The primary pathways that emerge out of these studies are the Notch, AKT-PI3K-mTOR, arachidonic metabolism, MMPs, STAT3, and EGFR, which need to be investigated further for their applicability. Validation of these markers/pathways in patient cohorts is essential to establish their clinical benefit and thereby adopt them into the concept of personalized medicine. Randomized patient trials that enable administration of drugs based on molecular profile and observational studies that correlate the marker status with the clinical outcome will help establish a panel of clinically viable biomarkers.

12.2 Bioinformatics

In the era of personalized medicine, oncologic treatment decisions are being made considering the patient's clinical presentation in the context of their genomic information. As discussed previously, a wide spectra of genomic aberrations, such as mutations or single-nucleotide polymorphisms (SNPs), copy number changes, structural variations, and gene fusions, are known to cause cancer. A personalized therapy approach must therefore be able to accurately determine the nature of the causative alteration present in a patient's genome. Currently most of the personalized clinical testing relies on NGS-based cancer panels for variant detection.

A cancer panel contains a selection of genes known to be involved in or having relevance to cancer. Targeted panels allow for identification of actionable mutations in a patient's tumor DNA as well as enable discovery of novel cancer-associated variants. Depending on the manufacturer, various commercial panels are available to assess high-risk cases with respect to somatic and germline mutations [67]. Compared to whole-genome or whole-exome sequencing, cancer panels are preferred for personalized medicine as they generally offer faster turnaround time and relatively higher coverage needed for accurate variant detection even at low frequencies [68, 69]. However there are several bioinformatics challenges associated with NGS-based personalized therapy, which if not addressed would lead to confounding and inaccurate information. The challenges exist across the entire workflow due to varied sample characteristics and various implicit assumptions of data processing and SNP detection algorithms. It is therefore important to be cognizant of the inherent complexities present in every step of the variant detection pipeline in order to avoid misinterpretation of results.

Several factors impact the accuracy of the variant detection, resulting in false-positive and false-negative SNP calls. These include but are not limited to stromal contamination in biopsy samples, clonal heterogeneity, improper handling during creation of formalin-fixed paraffin-embedded tissue (FFPE) blocks, sequencing

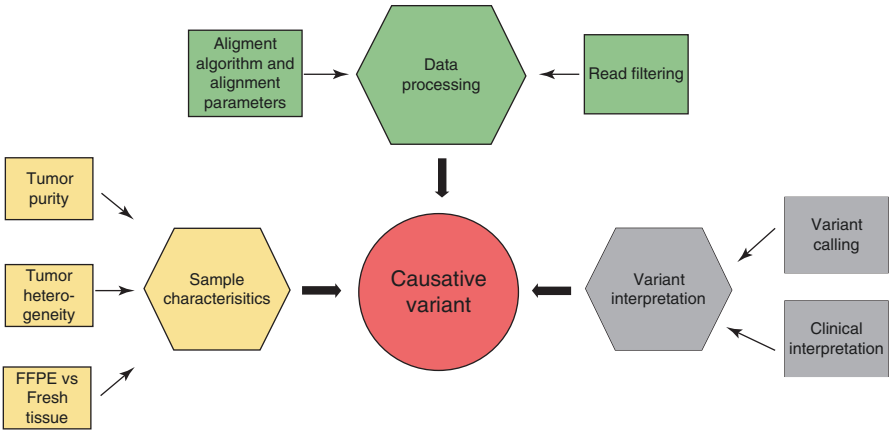

Fig. 12.1 Factors affecting the sensitivity of detection of causative variants [1]. Sample characteristics-sample purity, heterogeneity, and sample storage characteristics determine the accuracy of variant calling. Data processing and variant interpretation are other confounding variables

errors, and low throughput leading to insufficient reads and coverage. Broadly the accuracy of variant detection is dependent upon (1) sample characteristics, (2) processing of sequencing data, and (3) interpretation of the variant calls. This algorithm is shown in Fig. 12.1.

12.2.1 Sample Characteristics

12.2.1.1 Quality and Quantity of DNA

The first challenge to the design and development of any genomic assay in solid tumors lies in the biopsy sample itself. While fresh tissue biopsies are most suitable, a majority of tumor specimens that are available for any genomics assay are FFPE and are archived in various hospitals and pathology labs. Biopsies are often a challenge both for their quantity and quality. Sequencing often fails due to availability of limited amount of available tissue (core biopsy, fine-needle aspiration biopsy), thereby limiting the quantity of DNA that can be extracted. Most DNA-based tests require 200–1000 ng DNA as input material. However, a majority of samples in head and neck cancer are surgical specimens where there is adequate tissue available. In these cases, limiting tissue situation arises when a block is already depleted by multiple histopathology tests and then arrives at the lab for genomics testing. Another situation often encountered is when a large fraction of cells in the specimen tissue are necrotic or apoptotic cells, which yield low DNA quantity.

An even bigger challenge is extracting nucleic acids from FFPE specimens. DNA or RNA integrity is severely compromised in these tissue blocks often due to poor archival conditions or improper fixing at the point of making the blocks [70]. These result in smaller fragments of DNA (Fig. 12.2) as well as DNA-DNA

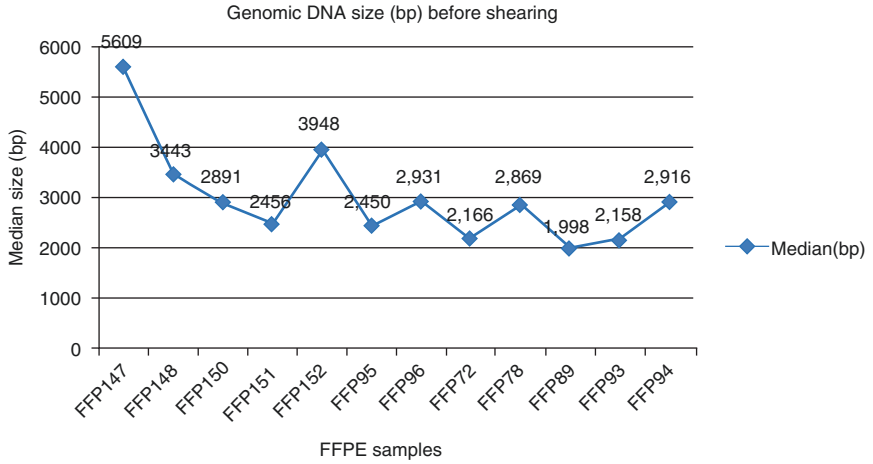

Fig. 12.2 Average genomic size of 12 diverse FFPE samples. The extracted DNA yield is considerably smaller fragments (2–6 kb) as opposed to standard 15–25 kb genomic fragments from intact tissue, blood, or saliva

cross-links that make certain region inaccessible to a probe or the polymerase during either hybridization or amplification.

In turn these regions are inadequately represented in the sample libraries in an NGS assay. Deep sequencing of these samples would yield poor coverage or inadequate number of sequencing reads in certain regions of the genome and consequently a higher fraction of low-coverage regions. Generating a larger number of reads for that sample using the Illumina Hiseq over a Miseq (http://www.illumina.com) or multiplexing fewer samples together in a sequencing run can partially address the issue of inadequate reads, but not completely.

Another peculiarity of the FFPE sample quality is the high percentage of non-reproducible mutations introduced into the FFPE sample due to cytosine-cytosine cross-linking whereby the Taq polymerase cannot access the site and inserts a T in place of C or A in place of G [71]. These are non-distinguishable from actual C>T mutations in the tumor which are actionable. However, these C>T conversions are low-frequency random events and not reproducible. Samples where these occur are distinguishable by the extraordinary high number of mutations.

False-positives can be eliminated by a second round of sequencing and considering only reproducible mutations for downstream analysis. Most of these issues are not relevant for fresh tissue samples.

12.2.1.2 Sample Purity: Normal Cell Contamination and Tumor Content

A tumor microenvironment contains several types of nonmalignant cells, and therefore tumor biopsies often contain adjacent residual normal cells or infiltrated normal cells [72]. However, a high normal cell contamination would eclipse the true mutational and copy number signatures of the tumor DNA. Thus it is very important

to ensure that the sample has limited nonmalignant normal cells, with a tumor content of up to 60 % usually considered acceptable. It is also important to note that the impact of normal cell contamination is critical only while identifying somatic mutations and does not impact the germline mutation detection. Mathematical models exist which estimate and incorporate the normal cell contamination in variant calling algorithms [73]. Methylation content of the tissue section has also been suggested as an indicator of normal cell contamination, since specific regions of the genome are methylated only in tumor cells and not in normal cells [74].

12.2.1.3 Tumor Heterogeneity

A major challenge posed by the tumors to the identification of somatic mutations is presence of intra-tumor heterogeneity or clonal diversity. As a result of heterogeneity, different tumor populations within the same tumor may harbor different mutations. In a study, biopsy from three different regions of an oropharyngeal carcinoma and two regions of the lymph node metastasis showed that the regions shared only about 41 % common single point mutations [75]. This leads to two different challenges. Firstly, heterogeneity implies that a single biopsy may not be sufficient to conclude treatment decisions. Secondly, the mutations of interest would be low-frequency mutations which would be difficult to detect. Detection of low allelic frequency somatic mutations, therefore, requires deep sequencing to be performed to ensure that the mutation has enough read coverage so that the variant can be called with high confidence. The extent of deep sequencing required would depend upon the extent of tumor heterogeneity and the desired sensitivity. Current algorithms can discern mutations present at an allelic frequency of 5 %.

12.2.2 Processing of Sequencing Data

Apart from sample characteristics, processing of the NGS data has a significant impact on variant detection. Broad steps in data processing involve (1) alignment against a reference, (2) filtering to remove low-quality reads, and (3) variant detection.

12.2.2.1 Alignment

Variant detection is done by comparing the patient DNA against a reference genome. As a consequence of this process, several parameters like alignment score, read mapping, base quality, etc., can affect the outcome of variant calling. In an initial step, reads obtained from a sequencing run are aligned against a standard reference sequence (HapMap or a person's own normal DNA). Mis-alignment and wrong mapping of reads at this step result in false mutation calls. If a correct alignment algorithm and appropriate alignment parameters are not chosen, reads can get improperly aligned due to the presence of mutations and indels in the genome.

Most of the personalized medicine sequencing is done for a small set of genes, i.e., the cancer panels in which the alignment can be done in two different ways. The sequence reads can be aligned to the entire genome or can be aligned only to the

regions of interest which are present in the panel. For amplicon-based panels, where only 2–3 % of total reads lie outside the defined target regions, typically alignment is done only to the target regions. However for probe capture-based enrichment panels, the situation is slightly different. Given that the probe has a very high probability of hybridizing to homologous regions of the genome that share 90 % or more sequence similarity, it is recommended that reads be aligned against the whole reference genome, rather than to the target region [76]. This is because when reads are aligned to only the target regions, partially overlapping reads which have originated outside the region may be missed. In addition, homologous reads aligning at a position from which they did not originate lead to mismatches which would be wrongly called as mutations. Another disadvantage of this approach is that it would cause misclassification of reads which are multiply mapping. Multiply mapping reads are defined as reads which map to multiple places (typically regions of high sequence similarity), which makes determining its actual origin difficult. Such reads are typically discarded and not carried over for further downstream analysis. Instead, reads mapping to a unique position in the genome are considered. By forcibly aligning reads to a defined sequence of targets, there is a danger of multiple mapping reads classified as being uniquely mapped. In a recent study, such errors resulted in an extra 88 % SNP calls, of which an overwhelming number (nearly 92 %) were false-positives [76]. The same study also designed another algorithm to successfully align reads to only the target regions, thus decreasing the computational time significantly.

Apart from the sequence used for alignment and the choice of alignment algorithm, alignment parameters play a critical role in germline and somatic mutation detection [77, 78]. Sensitivity, specificity, and limit of SNP and indel detection are dependent on the percentage of mismatches and number of gaps allowed during alignment. For short read (150 bases length) alignment, modulation of percentage mismatch and gaps allow for detection of even 40 bp deletion or insertion event. With decrease in alignment stringency, the number of events detected during variant calling increases with a concomitant increase in false-positive calls. Number of mismatches allowed per read would also determine the number of SNPs that can be detected within a read. Thus if number of mismatches allowed per read is one, then SNPs which are closely spaced would not be detected. While germline aberrations are present in all cells, somatic mutations are present in a subset. Therefore, for somatic mutation detection, it is important to lower the alignment stringency.

12.2.2.2 Read Filtering

Given all the challenges in detecting true variant calls, it is essential that variant calling be done using an unambiguous read list. The following read quality parameters should be considered before using reads for variant calling.

Alignment Score A good alignment score indicates less number of mismatches of the read to the reference sequence, where as a mismatch indicates the possible presence of a SNP. Technology-related errors often show up as mismatches which are difficult to separate from true biological SNPs. Presence of large number of

mismatches within a single read would indicate the read to be of questionable quality, and therefore SNPs detected only by reads with several mismatches throughout the read length should be discarded. A stringent approach for reducing false-positives would be to use an alignment score of 98 which would have the ability to detect the presence of up to 3 SNPs in a region of 150 bases. For clinical applications, an additional preferred approach would be to use a lower stringency alignment score of 95 to account for technology-related errors like PCR-introduced artifacts. Additional parameters like annotation against databases and strand bias are used to distinguish real variants from false-positives.

Mapping Quality After alignment, reads are assigned mapping quality scores which indicate the measure of confidence that the read originated from the position to which it has been mapped by the alignment algorithm [79]. Because the human genome contains repetitive regions, reads from such regions can map equally well to the multiple repeat positions. Sequencing errors resulting in a base change may also cause a read to map to multiple positions. Reads with low mapping quality and mapping onto multiple positions in the genome introduce coverage artifacts and should therefore be removed before proceeding with variant calling.

Base and Read Quality Base quality indicates the probability that the base is wrongly called. A read quality is an average of all base qualities present in the read. Reads with low quality will increase the false SNP calls and therefore should be discarded.

Duplicate Reads One of the huge sources of artifacts in sequencing comes from presence of duplicate reads which arise as a result of PCR bias or due to poor quality of DNA. Duplicate reads do not accurately represent the genome complexity, do not provide unique information, and can inflate supporting read percentage for variant calling, leading to false-positive calls [80]. An important aspect to consider while removing duplicate reads is that while the duplicates are easily identifiable in capture-based target enrichment protocols, it is not possible to distinguish duplicate reads in amplicon-based protocols. Duplicate reads are therefore retained while using amplicon-based panels, and variant calling is done using an extremely high coverage of 500–1000× to offset the false-positive rates due to presence of duplicate reads. A newly emerging single-molecule tagging (SMT) technology will allow identification of PCR duplicates in amplicon-based deep sequencing data [80].

12.2.3 Variant Prioritization and Clinical Interpretation

Variants observed in the target genomic locations should be evaluated for their clinical significance in order to pinpoint the causative variants. The next big challenge is to filter out unreliable variants and to assess the pathogenicity of the remaining SNPs. Variant prioritization is usually done using the following parameters.

12.2.3.1 Coverage and Percentage Supporting Reads

Coverage indicates the total number of reads covering a given locus, while supporting reads are defined as reads which support the presence of the alternate allele. Good coverage and presence of large percentage of supporting reads are critical to a confident variant call, especially for low-frequency alleles. Variants below <10 % are usually considered sequencing artifacts, but with sophisticated SNP-calling algorithms using priors, SNP can be called even as low as 1–2 % with 95 % or higher confidence.

Due to the impact of duplicate reads on variant calling, coverage considerations are different for amplicon-based and capture-based enrichment panels. While coverage of 300× is good for capture-based enrichment panels, amplicon sequencing requires 500–1000× coverage for a reliable call.

Uneven coverage across target regions is a major consideration as it leads to regions with zero or very low coverage. For cancer samples, uneven coverage across target regions is thought to be caused by genetic variations in cancer genome which interfere with hybridization [81]. Filtering multiply mapping reads also introduces a coverage artifact. For regions of low coverage, Sanger sequencing can be used to validate the identified variants.

C to T and G to A Artifacts Cytosine deamination leading to C to T transitions in FFPE samples is very common. Care should therefore be taken in prioritizing such mutations and they should be evaluated in the context of overall C to T rates. If overall C to T rates are very high, the sample should be re-sequenced and only those SNPs which are being called reproducibly should be considered.

Strand Bias Strand bias is a phenomenon where genotype calls inferred from forward and reverse strands are in disagreement with each other, e.g., reads mapping to forward strand display heterozygosity while reads mapping to the reverse strand display homozygosity [82]. Strand bias does not display any consistent pattern or preferred loci and occurs randomly and is thought to be caused by sequencing library preparation artifacts. Another type of strand bias which leads to unbalanced read mapping to forward and reverse strands is an artifact of exome capturing mechanism. SNPs with extreme strand bias are more likely to be false-positives and should be discarded.

12.2.4 Annotation Against Databases

12.2.4.1 Novel Versus Known Variants

Known variants refer to SNPs which have already been identified and catalogued in databases such as dbSNP and COSMIC (Catalogue of Somatic Mutations in Cancer) [83, 84]. Comparing the function of the known SNPs, as reported in the databases with the clinical manifestations, provides a very useful way of prioritizing variants identified in a tumor sample. COSMIC is a resource for somatic cancer mutations, and thus patient mutation which has been reported in COSMIC with a very high

frequency indicates that it is probably very relevant. On the other hand, dbSNP contains all SNPs submitted to the database irrespective of their pathogenicity. dbSNP is also not exclusive to cancer and contains SNPs relevant to other disorders as well. If the patient SNP has been reported with a high allele frequency in a population and with no clinical significance, then most likely it would be a germline SNP or a somatic SNP with no role in cancer.

Novel SNPs require more thorough investigation and very careful consideration before they are established as functionally damaging and being clinically relevant. The novel SNP is usually interpreted in the context of its location in the genome (see subsection location below) and the impact on protein structure and function.

12.2.4.2 Somatic Versus Germline

Mutations can be unambiguously assigned as germline or somatic only when a paired normal analysis is done, i.e., sequence of the tumor is compared with the sequence from the "normal" or non-malignant tissue of the same patient, usually blood or saliva (in case of oral cancer, saliva should not be used as the source of normal cells). Establishing a mutation as being germline or somatic has important diagnostic and therapeutic implications. Cancer origin, progression, and metastatic spread can be traced to the somatic mutations which are localized to the tumors. If found to be actionable, tumors with somatic mutations can be evaluated as candidates for targeted therapy. Germline mutations are indicators of a patients' susceptibility, since they are present in every cell and are not actionable. Germline mutation information is used for risk prediction, prophylactic measures, and aggressive screening of family members.

Matched normal sequencing increases the patient burden in terms of cost and of late tumor-only sequencing approaches are being employed. Using specific criteria SNPs are identified as likely being germline or somatic. A recent study has cautioned against the use of tumor-only sequencing method, as it led to 31 % increase in false-positives, i.e., germline mutations in actionable genes were referred to as somatic [85].

12.2.4.3 Synonymous Versus Non-synonymous

Mutations can be synonymous or non-synonymous. Since the synonymous mutations do not impact the protein structure, they are generally not considered for further analysis. Impact of the non-synonymous mutations is assessed based upon their location and pathway relevance and whether it is actionable.

12.2.4.4 Location

SNPs can be present in locations which would have a significant protein effect, either in terms of its structural integrity or regulation. Location of the mutation many times determines whether it is actionable. Mutations which result in a gain of stop site and truncation, a frameshift, or changes in essential splice sites are considered critical, especially if it happens in the first few exons. Mutations in upstream and downstream regulatory regions have more regulatory effects.

12.2.4.5 Pathway

Any change, be it a mutation or any structural variation (amplifications, deletions, translocations) in a cancer-related gene, should not be viewed in isolation but with its impact on cancer-related pathways. Thus targeted therapy against an actionable mutation cannot be administered if there is a downstream mutation bypassing its effects.

In summary, variants present in a patient first have to be identified and prioritized in order to establish their clinical significance. Variant can usually be evaluated using several criteria in parallel, and each of these has different degrees of experimental, computational, algorithmic, and interpretation challenges associated with it, which determine the outcome [22]. Each SNP has to be viewed in the context in which it was identified. For example, a C-to-T from an FFPE has to be considered taking into account the overall sample performance. Similarly, a mutation present in the first or second exon leading to protein truncation will be more clinically significant than a truncation in the last exon. Again, targeted therapy for a clinically significant actionable mutation can be given only if there are no other damaging mutations downstream. Eventually, all the processes in a clinical pipeline, i.e., preparation of sequence library, choice of reference genome used, alignment parameters, algorithmic assumptions, a priori biological knowledge for clinical interpretation, and relationship of a damaging variant to a patient's symptom, impact the quality of decision making and patient management.

12.3 Personalized Medicine

12.3.1 Expression Profiling in Head and Neck Cancer

The use of the mRNA profile to classify tumors and hence inform treatment is not a new concept. Well-known products in the market such as Oncotype DX® or MammaPrint have been used over the past decade to stratify breast cancer patients, estimate risk of recurrence, and decide whether chemotherapy is needed. There have been similar attempts to understand chemotherapy sensitivity in head and neck cancer as well. Higuchi et al. compared the gene expression profiles between an HNSCC line sensitive to cDDP and its cDDP-resistant variant to develop a 5-gene signature of cisplatin resistance in HNSCC [86]. Since radiotherapy is a major treatment modality in nasopharyngeal cancer, Chang et al. established two radioresistant subclones from NPC parental cell lines by treating the cells with sublethal ionizing radiation [87]. Comparing the expression profiles of the resistant cell lines with their parents, they identified a 7-gene signature of radioresistance. Using siRNA, they also showed that interfering with these genes made the clones more susceptible to radiation. In another study, Ganly et al. set out to identify a signature related to locoregional failure in patients with laryngopharyngeal cancer undergoing chemoradiation therapy (CRT) [88]. They collected tumor tissue from patients who had undergone CRT, studied their expression profiles,

and compared them with their treatment outcomes. This approach allowed them to develop a 17-gene signature that correlated with locoregional failure in laryngopharyngeal cancers.

While many of these approaches may be promising, the expression-based approach has various limitations largely based upon the instability of the mRNA and the difficulty in creating a robust assay. Since the transcriptomic profile is partially determined by the mutational pattern in the tumors, it makes clinical sense to work at the DNA level and use deep sequencing to profile tumors.

12.3.2 The Mutational Landscape of the Tumor

As discussed in a previous section, the inherent molecular complexity and heterogeneity of cancer provides the rationale for studying the mutational profile in a tumor. Identification of genes that drive tumorigenic pathways in an individual's cancer can potentially provide personalized therapy options. When this approach began more than a decade ago, it consisted of genetic testing for driver mutations in a single gene, followed by treatment with therapies to target specific pathways essential to the growth and spread of that cancer. These therapies provided a more effective and less toxic treatment options than conventional chemotherapy and radiotherapy. However, it started becoming increasingly apparent that in many cases, detection of mutations in a single gene alone was not sufficient. Cancer cells exhibit multiple layers of redundancy and cross talk, making single-pointed interventions insufficient for many individuals positive for "driver" mutations. This complexity makes it necessary to both take a larger view of the multiple pathways involved and a deeper look at the genetic makeup of the tumors.

12.3.3 The Role of Next-Generation Sequencing

With the advent of improved sequencing technologies such as NGS, profiling a tumor to detect therapeutically relevant mutations has become increasingly viable. NGS-based tests can typically profile a several hundred genes causally implicated and clinically relevant in cancer. This deep sequencing technology can detect mutations with far greater sensitivity than other conventional sequencing methods, thus making it ideal to study tumors, although its application in head and neck cancer is not advanced as in other cancers.

The systematic cataloguing of cancer mutations by large institutes such as the Sanger Institute and consortia such as the International Cancer Genome Consortium (ICGC) and the Cancer Genome Atlas (TCGA) has facilitated the identification of genes and mutations most associated with head and neck cancer [7]. According to the COSMIC database, the most frequently mutated genes in head and neck cancer include *TP53, CDKN2A, PIK3CA, MET, HRAS, EGFR, PTEN, BRAF, KRAS, PIK3R1, IL6ST, JAK3, NFE2L2,* and *FBXW7.* Many of these genes drive the cancer

process either singly or in combination, and assessing the role and impact of their mutations can yield therapeutically relevant insights. Certain mutational profiles could indicate poor response to a particular regimen that would be typically considered for the patient. Some mutations may be indicators of overall prognosis or response to certain types of chemotherapy. Interestingly, some of these mutations could indicate response to a different drug regimen not typically considered in head and neck cancer, thus uncovering new therapeutic options. It is increasingly being appreciated that tumors of different tissue origins can have the same driver mutations and hence can be targeted using the same drug. As an example, cetuximab, originally approved by FDA for colorectal cancer, has more recently been approved for head and neck cancer as well. The idea of repurposing other drugs supports the idea of testing a tumor for mutations in genes other than the ones most commonly associated with that tumor. Thus a multigenic profiling of a tumor can help build a potentially actionable mutation landscape, allowing the treating oncologist to most efficiently arrive at the most effective therapy plan. A consequence of this paradigm shift in thinking is that cancer treatment is now being actively investigated based upon the underlying mutational profile, leading to drugs approved for certain cancers being considered and clinically tested in trials for other cancer types.

It is known that greater than 90 % of the head and neck cancer patients overexpress EGFR, either via gene amplification or due to polymorphic mutations [89, 90]. Thus anti-EGFR drugs provide a viable therapeutic option. As discussed previously cetuximab for treatment of advanced head and neck cancers reported improved survival of head and neck cancer patients who used cetuximab in combination with either platinum-based chemotherapy in the recurrent or metastatic setting or with radiotherapy for patients who are not able to derive benefit from platinum-based agents [91, 92]. Although the results from the clinical trials are promising, the picture can get complicated quickly. Firstly, overexpression of *EGFR* results in higher levels of activated EGFR, leading to increased activation of the downstream RAS/RAF/MEK/ERK1/2 signaling pathway, stimulating cell proliferation [93]. While such tumors are ideal for treatment with cetuximab, the presence of any additional activating mutation in the pathway downstream such as codon 12 or 13 mutations in *KRAS* will make the tumor refractory to anti-EGFR drugs. Head and neck cancer patients harboring mutations in the RAS/RAF/MEK pathway will probably fail to respond to cetuximab. Adding a treatment that inhibits downstream proteins, such as MEK inhibitors, trametinib, or selumetinib, might be beneficial [94, 95]. Secondly, various clinical studies have reported that after the early response to cetuximab, head and neck tumors gradually acquire resistance to treatment [96, 97]. In such cases, the tumor mutation profile can help in planning a second line of treatment or a more aggressive first-line treatment. Head and neck cancer patients often harbor activating mutations in *PIK3C, PTEN,* or *AKT1*, resulting in constitutive activation of the PI3K/mTOR/AKT pathway. Given this, the tumors could possibly be sensitive to mTOR inhibitors such as everolimus or temsirolimus. Various clinical trials are currently underway to assess the efficacy of mTOR inhibitors and AKT inhibitors either as monotherapy or in combination with cetuximab or chemotherapy in head and neck cancer patients [98].

12.3.4 Clinical Correlation

As discussed, in addition to the lack of targeted therapies, another major barrier for reducing morbidity and mortality from HNSCC is the lack of an accurate measure of disease burden and response. Early detection, monitoring disease burden during treatment to confirm efficacy of surgery, radiotherapy and/or chemotherapy, and surveillance for early detection of persistent or recurrent disease are the optimal approaches for reducing morbidity and mortality from this disease. Current imaging methods make assessment of early disease, response to treatment, and differentiation between progression and treatment effect very challenging. Moreover, clinical decision making is compromised because of the lack of accurate monitoring methods. Oncologists are forced to be reactionary and respond when disease burden is greater when the tumor becomes palpable or visible by examination and imaging.

12.3.4.1 Current Therapeutic Paradigm for HNSCC

The treatment of HNSCC depends on the site and stage at presentation. Oral cavity SCC tends to be treated with surgery followed by adjuvant chemoradiation, as indicated. Early-stage oropharynx, hypopharynx, and larynx tumors can be treated with single modality therapy composed of either surgery or radiation. However, up to 50 % of head and neck cancer patients present with advanced-stage disease. Advanced-stage disease involving these sites is treated with combination therapy including concurrent chemotherapy and radiotherapy or surgery followed by adjuvant radiotherapy +/− chemotherapy. Despite advances in our understanding and treatment of HNSCC, there has not been significant improvement in survival with a 5-year survival of approximately 50 %.

12.3.4.2 Methods for Monitoring Response and Progression of HNSCC

Despite multimodality therapy, local, regional, and distant recurrence is a major problem with recurrence rates reported to be as high as 65 %. Patients with locoregionally recurrent disease may benefit from salvage surgery and/or reirradiation +/−chemotherapy although survival tends to be poor. However, recurrences that are detected early are more likely to be successfully salvaged.

There is no consensus in the literature on the optimum frequency, duration, and interventions/studies for surveillance after treatment of HNSCC [99]. In general, physical examination with endoscopy, anatomic imaging, and metabolic imaging are used without uniformity. The same studies are used to determine response to treatment. However, due to the morphological changes due to treatment effect of prior therapy, the interpretation of physical examination findings and/or imaging data can be extremely challenging. There are no blood tests or serum tumor markers that are available to monitor response or progression of HNSCC.

12.3.4.3 Challenges in the Treatment and Monitoring of HNSCC

Treatment-induced anatomic and imaging changes often do not reflect actual changes in tumor size and can lead to premature and inappropriate changes in

treatment. Particularly challenging is the interpretation of imaging after radiation treatment or surgery when posttreatment changes are often indistinguishable from actual tumor progression. Currently only repeat biopsies or watchful waiting with repeat scans can help in making this distinction.

Conclusion

The notion that each patient with his/her tumor is unique and needs a treatment that is personalized is beginning to find widespread application especially in the case of cancer. In modern medicine one could classify patients based upon molecular signatures in tumor cells, which differ not only from one patient to another but also within a patient, where the tumor genetic diversity is large. The major need to classify patients is that if one is able to understand the molecular alterations in a patient that drive the malignant transformation of cells leading to unrestrained cellular proliferation and establishment of cancer, one could design diagnostic and therapeutic strategies to intervene and stop these processes, leading to disease arrest and eventually a cure.

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Role of Cancer Stem Cells in Oral Cancer

13

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13.1 Introduction

13.1.1 Cancer Stem Cell Concept in Carcinogenesis

The involvement of stem cells in cancer has been hypothesized over the last several decades; however, actual proof for the hypothesis of cancer stem cells (CSCs) has been established only over the last few years. Today, CSCs are defined as rare cells in tumors with indefinite potential for self-renewal that drives tumorigenesis [1]. Isolation of CSCs in acute lymphocytic leukemia through extensive cell cloning marked the first evidence for the presence of CSCs. This work was done by John Dick and co-workers who successfully demonstrated the critical property of stem cell –self-renewal in association with a cell fraction that constituted only about 1 % of the tumor but were the only cells capable of generating a new tumor in immunocompromised mouse models [2].

Two models have been put forth to describe tumor formation from CSCs, viz., the stochastic and hierarchial models. The former postulates that each cell within the heterogeneous tumor has an equal but extremely low tumorigenic potential [3]. In such a case, tumor progression is a continuous process involving positive selection of genetically unstable clones that confer survival to a tumor within the prevalent microenvironment. This model accounts for the emergence of drug resistance during chemotherapy as an adaptive process through selection of cells with genotypes that allow survival from drug exposure [4]. Isolation of progenitors is not

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reproducible using this model as their existence is random. Alternatively, the hierarchial model puts forth that tumorigenic potential is limited to a very small clonogenic population of cells within tumors that define CSCs. Commitment of a CSC to the regenerative process produces a hierarchy of cells at different stages of differentiation. This model suggests that CSCs form a rare yet distinct subset of cells, while the large majority of tumor cells represent descendants of these CSCs that progressively lose their self-renewal capacity [1].

13.1.2 Normal and Cancer Stem Cells

A tumor may thus be viewed as an aberrant organ initiated by a CSC that has acquired the capacity for indefinite proliferation through accumulated mutations. In such a scenario, the principles of normal stem cell biology can be applied to understand how tumor develops as a defective regenerative process. Several observations suggest that analogies between normal stem cells and tumorigenic cells are appropriate. Normal tissues are composed of heterogeneous cell types that have different phenotypic and functional characteristics and different proliferative potentials. Since most tumors are considered to be clonal, CSCs must be able to give rise to phenotypically diverse progeny, including cells with indefinite proliferative potential, as well as cells with limited or no proliferative potential. This suggests that tumorigenic cancer cells undergo processes that are analogous to the self-renewal and differentiation of normal stem cells.

13.1.2.1 Self-Renewal

Both normal and cancer stem cells exhibit the property of self-renewal which can be defined as a capacity to undergo asymmetric division that generates a quiescent stem cell and a committed progenitor. The latter further contributes toward developing the critical mass of cells required for regeneration [5, 6]. Epigenetic regulation of stem cell properties is now being understood [7]; self-renewal in stem cell types at the epigenetic level is by polycomb genes (BMI-1 and EZH2) [8, 9], and also signaling pathways (Wnt, Sonic Hedgehog, and Notch) [10]. Functional plasticity is induced through reacquisition of pluripotency and immortality that in turn is driven by transcriptional factors such as Oct4, Nanog, and Sox2 [11]. Recently, Skp2, a component of the Skp2-SCF complex, has been suggested to be an important regulator of HSC quiescence, frequency, and self-renewal capability. Skp2 deficiency displays a marked enhancement of HSC populations through promoting cell cycle entry [12].

13.1.2.2 Proliferation

Both normal stem cells and CSCs have extensive proliferative potential and exhibit enhanced telomeres and telomerase activity that extends doubling capacity and cellular life span. Surface expression of ABC (ATP-binding cassette) transporters contributing to cellular resistance against specific growth inhibitory drugs is another capability that is shared by these cells [13] as also is predisposition of growth factor independence acquired through autocrine secretion of growth factors and cytokines.

13.1.2.3 Metastasis

Expression of surface receptors like CXCR4, CD133, α6 integrin, c-kit, c-met, and LIF-R identified as stem cell markers are also associated with homing and metastasis, which is a characteristic of both normal and cancer stem cells [14–18]. While homing in the normal scenario is toward repair and regeneration of damaged/depleted tissue, in the case of transformation, it becomes a mechanism of tumor survival by migrating to newer and more amenable niches when the primary tumor site becomes limiting due to depletion of nutrients.

13.1.3 Initiation of Carcinogenesis

Research over the last decade has attempted to associate cellular mechanisms with mutagenic effects within tissues as a causative event leading to the emergence of CSCs. The various events that could be involved include the following:

13.1.3.1 Stem Cell: Target of Transforming Mutation

A multipotent tissue stem cell is subject to DNA damage and repair events throughout its life span. Accumulation of such aberrant events in a single stem cell may lead to transformation. The phenomenon is also supported by disruption of the stem cell niche with a shift toward growth-promoting signals rather than growth-inhibiting signals. This results in a state of frequent stem cell activation that ceases to be a transient regenerative mechanism required for normal tissue homeostasis. Excessive hormonal stimulation, recurrent post tissue damage, inflammation, radiation, chemicals, infections, inactivation of tumor suppressor genes, or activation of oncogenes may provide the stimuli for the former state [19]. Studies with human prostate stem cell self-renewal and differentiation by natural steroids as well as EDCs (endocrine disrupting chemicals) support the hypothesis that tissue stem cells may be direct EDC targets and can undergo lifelong reprogramming as a consequence of developmental and/or transient exposures [20].

13.1.3.2 Progenitor Cell: Target of Transforming Mutation

Alternatively, it has been suggested that transiently amplifying (TA) progenitors that are relatively uncommitted progenitor cells may undergo transformation following a series of oncogenic mutations that lead to CSC generation. Since progenitor cells are directly derived from stem cells, the process requires minimal genetic alterations to reacquire the critical stem cell properties.

13.1.3.3 Dedifferentiation of a Differentiated Cell

A committed progenitor or differentiated cell may undergo a phenomenon of dedifferentiation to acquire stemlike properties. This phenomenon is widespread in plants and to some extent in lower animals [21–23]. The recent euphoria over transfection of a "cocktail" of genes that transforms fibroblasts into cells with stem cell-like properties now suggests that the phenomenon may be achievable and be applied for therapeutic purposes. The stem cell properties thus reacquired by

differentiated fibroblasts through reprogramming include the ability to self-renew and differentiate along multiple lineages. On the flip side unfortunately, its aberrant reprogramming may result in the generation of CSCs [24, 25].

13.1.3.4 Fusion of Tissue-Specific Stem Cells with Circulating Bone Marrow Cell

A CSC may also be generated through fusion of bone marrow-derived stem cells with circulating differentiated cells. This is believed to involve the mobilization of bone marrow-derived cells either at an inappropriate time or place within other tissues leading to transformation and acquisition of a stemlike phenotype [26] and fusion of mesenchymal stem cells with lung cancer cells induced CSC-like properties in the hybrid cells [27]. Several CSCs are known to express both pluripotency and self-renewal markers characteristically expressed on hematopoietic stem cells, making it a vital possibility although the link has not been clearly established.

13.1.4 The Niche Concept

The niche concept introduced by Schofield et al. was largely neglected until *Drosophila* studies provided a stimulus for its resurgence [28]. A niche can be defined as a specialized local tissue microenvironment capable of housing and maintaining one or more stem cells. Thus, a stem cell niche is an interactive structural unit organized to facilitate cell fate decisions in a proper spatiotemporal manner. Niche cells provide a sheltering environment that sequesters stem cells from differentiation or apoptotic stimuli besides other environmental triggers that would challenge stem cell reserves. Excessive stem cell proliferation may lead to cancer, and therein lies a role for the niche to maintain stem cells in a quiescent state by keeping a check on activation, proliferation, and fate determination mechanisms [29, 30]. Maintaining a balance between the proliferation and antiproliferation signals is key to homeostatic regulation of stem cells that permits self-renewal yet supports long-term tissue regenerative potential. Many developmental regulatory signal molecules, including Shh, Wnt, bone morphogenetic proteins (BMP), fibroblast growth factors, and Notch, have been shown to play roles in regulating stem cell self-renewal and lineage fate in different systems [31]. This indicates that the niche itself is, also, under dynamic regulation and any deviation may lead stem cells to become independent of growth signals that trigger uncontrolled proliferation and tumorigenesis.

13.1.5 Molecular Basis/Markers of Stem Cell Transformation

It is now equivocally accepted that tumors consist of mixture of self-renewing stem cells, transiently amplifying progenitors, and proliferative cells with a shorter life span that can undergo limited differentiation. Apart from these, several other cell populations including vascular and angiogenic populations and stromal and

myofibroblast cells contribute to tumor heterogeneity. Some of these may also have stemlike properties making isolation of CSCs a formidable task.

However, the last decade has focused on development of several strategies for CSC isolation. Essentially, these are based on knowledge of surface markers, expression patterns, and immunophenotyping of stem cells in the normal organ/tissue; the implied correlation is that even in the aberrant transformed state, these markers would still be valid. The isolation and identification of leukemia-initiating and tumor-initiating cells in other tumors such as breast and brain using multiparametric flow sorting and immunocompromised mouse models established such approaches, which are a landmark in CSC research since it demonstrated the involvement of these rare cells in the disease [2, 32–35]. Various surface markers are used for identification and isolation of CSCs across an array of tumor types for example: CD133+ (liver, prostate, colon, and neural cancer) [33, 36–39]; CD44 (pancreas, colorectal, and mammary cancer) [32, 40, 41]; EpCaM (colorectal cancer) [40, 42]; and CD34+, CD38−, CD96+, and CD90− (acute myeloid leukemia) [43–45]. Interestingly, most stem/CSC markers have been identified based on expression patterns without assigning any potential function to their "stem state." Their probable roles are diverse and include regulation of differentiation, homing, adhesion, establishment of cell polarity, and migration via cell-cell and cell-matrix interactions [46–49].

Considerable debate exists regarding the application of these surface markers toward isolation and identification. Expression of molecules involved in adhesion could simply be a reflection of a functional need of the assay itself. For this reason, it would be favorable to identify CSCs by markers that have a clear function in CSC biology. CD133 was initially identified as a marker for isolation of stem and progenitor cells of the hematopoietic system [50]; later on, it was identified as CSC marker in case of cancers like colon and hepatocellular carcinoma [38, 51, 52]. However, several labs including ourselves have demonstrated that CD133 expressing stem cells in cancer metastases could be non-tumorigenic but retain a potential to contribute to tumor vasculature by differentiation along the endothelial lineage and establishment of a classical stem cell hierarchy [53]. Retention of a functional capability to grow as xenografts and sequential maintenance of tumorigenicity over three to four cycles to demonstrate self-renewal mechanisms in experimental animals thus appears to be the most robust (although cumbersome) identification of stemlike regenerative activity in tumors.

In order to identify stem cells by exploring their property of quiescence, long-term label retention is widely used [54–56]. A standard label-retaining cell (LRC) assay exploits the slow-cycling nature of stem cells, whereas rapidly dividing, transit-amplifying (TA) progenitors generated through stem cell self-renewal progressive dilute their label intensity and after frequent divisions, the label is completely quenched [57]. Although bromodeoxyuridine labeling of DNA is frequently used in LRC assays, recent studies suggest it to be cytotoxic in nature [58]. Isolation of quiescent ovarian CSCs has similarly been reported using a membrane labeling fluorophore, viz., PKH-67. The fluorophore is retained by slow-cycling CSCs that undergo minimal divisions, whereas complete dye quenching is evident in highly proliferative differentiated cells [53].

13.2 Contribution of Cancer Stem Cells to Disease Progression

13.2.1 Angiogenesis

Survival and metastasis of tumors are often determined by their potential to establish efficient vasculature. Two possible sources of endothelialization are:

(a) Migration of endothelial cells and branching from preexisting vascular networks
(b) Recruitment of endothelial precursor cells (EPCs) from the circulation [59]

Circulating EPCs were originally identified in 1997 by Asahara as CD34 (+) VEGFR2 (+) mononuclear cells and demonstrated to provide both instructive (release of proangiogenic cytokines) and structural (vessel incorporation and stabilization) functions that contribute to the initiation of neo-angiogenesis. Recruitment of EPCs to sites of neo-angiogenesis is triggered by increased secretion of angiogenic growth factors or chemokines such as VEGF, angiopoietin, and stromal cell-derived factor (SDF-1) by tumors [60, 61]. Identification of such molecules, tissue-specific extracellular matrix components, and signaling pathways has provided new targets and therapies in cancer.

13.2.1.1 Role of Cancer Stem Cells in Angiogenesis

The synergy between CSCs and vascular niches is increasingly receiving attention with the evolution of a concept of the "CSC niche." CSCs provide proangiogenic factors to the developing tumor through niche to maintain tumor vasculature and are also sources of paracrine signaling and secretion of factors that promotes CSC self-renewal and maintenance (Fig. 13.1). In addition to a role in CSC maintenance, the tumor niche is hypothesized to be involved in metastasis by induction of the epithelial-mesenchymal transition, leading to dissemination and invasion of tumor cells [62–64]. Direct physical association between CSCs and endothelial precursor cells is also associated with enhanced tumorigenicity [65, 66]. Indeed, CSCs proactively recruit primitive vascular stem cells and endothelial precursor cells during metastases toward establishment of an effective tumor vasculature at secondary metastatic sites [67].

13.2.1.2 Vasculogenic Mimicry

Vascular mimicry is an adaptation exploited by some tumors in which CSCs retain a phenotypic plasticity, mimic endothelial cells, and form vascular channels that enable limited tumor perfusion independently of true angiogenesis [68]. Vasculogenic mimicry has been observed in melanomas, ovarian cancer, hepatocellular carcinoma, breast cancer, and glioblastoma [69–73]. The vascular endothelium in glioblastoma is reported to harbor a subset of tumorigenic cells that produced highly vascularized anaplastic tumors with areas of vasculogenic mimicry in immunocompromised mice [39]. As the ability of self-renewal and differentiation is

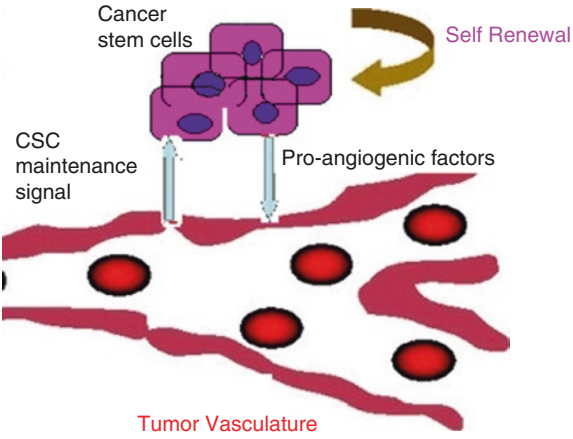

Fig. 13.1 Mutualistic association of cancer stem cells with tumor vasculature

restricted to CSCs within a tumor, the cells involved may be CSCs that transdifferentiate to generate vasculogenic derivatives and participate in tumor vasculogenesis [69, 74].

13.2.2 Metastasis

Invasion and metastasis of tumor cells are considered as highly inefficient since a very small fraction of the invading cells can actually regenerate secondary tumors. Hence, a necessary prerequisite for migrating tumor cells is high regenerative potential. Currently, the presence of two types of CSCs is suggested: (a) *stationary CSCs (SCSCs)*, restricted to the primary tumor, and (b) *migratory CSCs (MCSCs),* that emerge at the tumor-stroma interface and initiate invasion and metastases [75]. The phenomenon of epithelial-mesenchymal transition (EMT) identified earlier in association with embryonic development is increasingly being described as a characteristic of metastasis and is associated with invasive cancers. An important implication is that MCSCs may be derived from CSCs by acquisition of transient EMT – a fact that is well supported by data from some tumors but remains to be validated in several other tumors. Further, on reaching an amenable secondary site, a reversible process, viz., mesenchymal to epithelial transition (MET), is implicated in order to regenerate a tumor at this new site. Such reversible EMT-MET mechanisms represent a functional manifestation of cell plasticity and are believed to be fundamentally similar between normal stem cells (embryonic or adult) and CSCs [76]. These aspects have also led to reports that suggest EMT signatures to originate from certain subpopulation of CSCs and further be considered as markers of poor prognosis for such patients [77–80].

13.2.2.1 Signaling Pathways Involved in Stemness Also Induce EMT

The cues for transcriptional activation of the EMT circuits are relayed and regulated through Wnt, SHH, Notch, and growth factor-mediated signaling, which are also

involved in maintenance of stemness. In colorectal tumors and breast cancers, active Wnt/β-catenin signaling appears to be closely associated with cells undergoing EMT which are detected at the invasive front that also appear to undergo dedifferentiation [81–83]. A similar situation has been reported in endometrioid carcinomas and gastric, renal, and other cancers [83–85]

13.2.2.2 Transcriptional Regulators of EMT: A Potential Link Between Invasion and Stemness

Under conditions of stress in normal pancreatic epithelial cells, p53 regulates growth, EMT, and stemness [86, 87]. Current reports suggest that such regulation is possibly through modulation of specific miRNAs [88]. p53 thus emerges to be a major player in initiating EMT and stemness under conditions of stress. The involvement of several E-box binding transcription factors, including the Snail family members Snai1 and Snai2, Twist1, Zeb1, Zeb2, etc., has been extensively described in the context of regulation of cell proliferation, phenotype, migration, survival, and acquisition of stemlike cellular features [89]. In response to specific external cues, Snail represses cyclin D2 transcription leading to a G1/S cell cycle arrest that confers on cells a low proliferative potential yet permits their migration [90]. A mechanistic understanding of the process involved in Snail- and Slug-mediated radio- and chemoresistance has also been elucidated. Through a very elegant modulation of their target repertoire, these TFs have been shown to not only mediate EMT but additionally antagonize p53-mediated apoptosis and effect acquisition of a stemlike phenotype in ovarian cancer cells [91] and involve re-expression of Nanog and/or CD133 [92] and increased stemness characteristics in primary non-small cell lung cancer cell line [93].

Snai1 has been implicated in the recurrence of primary breast carcinomas [94]. Stable silencing of Snai1 in highly aggressive mouse epidermal carcinoma cell lines induces a dramatic reduction of tumor growth potential thus supporting its role in maintenance of the pool of regenerative cells. Increased expression of Twist1 also has been widely reported to have an adverse prognostic effect in various human cancers. Bmi1, a critical component of the polycomb repressive complex 1 (PRC1) which maintains self-renewal and stemness, is frequently overexpressed in several human cancers and can induce drug resistance. Twist1 directly activates Bmi1 expression and the two molecules function together to mediate cancer stemness and EMT [95]. Emerging evidence also suggests a role for Twist1 in expansion and chemotherapeutic resistance of CSCs [96, 97]. In post-EMT mesenchymal cells, Snail1 directly regulates Nanog expression, and loss of Snail1 reduces tumor growth without affecting tumor initiation [98]. This definitely indicates that EMT regulators mediate a potential link between invasion and stemness.

13.2.3 Chemo-/Radioresistance

Resistance to radiation and chemotherapy has been reported to be a defining characteristic of CSCs in various tumor types, including glioma, breast, and colon

cancers [99–103]. Various factors have been suggested to govern chemo- and radioresistance in CSCs:

(a) Expression of ABC (ATP-binding cassette) transporters (ABCG1 and ABCG2) is responsible for efflux of the drugs [13].
(b) Quiescent nature of CSCs facilitates them to escape classical chemotherapy which is often directed toward proliferative cells.
(c) CSCs are known to harbor lower levels of reactive oxygen species than the non-stem cell component that contributes to their radioresistance [104].
(d) Preferential activation of DNA damage response (in particular low proliferation and activation of the DNA damage checkpoint) promotes radio- and/or chemoresistance [99].

In order to overcome chemo- and radioresistance, different approaches have been tried including:

(a) Inhibition of angiogenesis within a tumor that would restrict development of tumor vasculature [105, 106]. This could be further enhanced if it could be coupled synergistically with traditional chemotherapeutic drugs.
(b) CSCs can be targeted through targeting of genes and transcription factor responsible for both chemo- and radioresistance. Reports do suggest the use of telomerase inhibitors can inhibit CSCs in different cancers [107, 108].

13.2.4 Tumor Heterogeneity

Therapy against cancer is largely ineffective due to heterogeneous, cellular nature of tumors that is also reflected at the molecular level. Cellular heterogeneity extends to virtually all measurable properties of cancer cells, including the size, differentiation state, proliferation rates, functionalities, migratory/invasive capabilities, and therapeutic responses. Such heterogeneity most likely represents a major therapeutic hurdle, but the mechanisms underlying its emergence remain poorly understood and controversial [109].

Recently, we have studied cellular heterogeneity within tumors with respect to stem cell-based proliferative hierarchies and varying ploidy levels. Tumors consist of hierarchies of cell populations based on proliferative potential of the cells (CSCs, progenitors, and differentiated cells). Isolation of these subsets using dye dilution has been demonstrated as discussed above. Additionally, the finding that 70 % of progenitor consists of aneuploid cells indicates that aneuploidy is another determinant that contributes to tumor heterogeneity and drug resistance [53]. Although the subsets identified on the basis of these two criteria are not mutually exclusive, the contribution of each now requires to be elucidated in order to study disease progression.

Molecular tumor heterogeneity is lucidly studied in breast cancer. The success of breast cancer subtyping based on a combination of molecular expression and

histology in guiding therapeutic strategies [110] has recently prompted similar investigations in other cancers including ovarian cancer [111]. In context of molecular heterogeneity, it is important to identify specific CSC-associated genes, from expression profiles of tumors consisting of heterogeneous population, as biomarkers and evaluate their correlation with patient survival. This could lead to an increased sensitivity and specificity of the prognostic/predictive value of these biomarkers. To date, several groups have identified gene expression "signatures" and biomarkers [112–114], but these may further need to be evaluated in the context of CSC biology.

13.3 Cancer Stem Cells in Head and Neck Squamous Cell Carcinoma

The concept of "stem cells" in head and neck cancer and their possible role in the initiation and progression of the disease has been the focus of investigations in recent times. The increasing interest has been due to the need to understand the process of head and neck carcinogenesis better with the ultimate objective of exploring possible clinical applications.

13.3.1 CSC Concept and Triggers for Transformation

13.3.1.1 Tumor Heterogeneity

As is observed with most solid tumors, head and neck cancers are extremely heterogeneous in terms of the cellular, vascular, and molecular content. Ninety-five percent of the head and neck cancers are known to be squamous cell carcinoma (HNSCC) suggesting a more homogeneous origin, but studies carried out down the years prove to be contrary. Categories of head and neck cancer patients with similar stage at presentation but distinct response to therapy and their survival/outcome are well documented. Cellular and histological subclasses of head and neck cancers that correlate to their clinical phenotype and outcome have also been identified.

Molecular heterogeneity attributed to head and neck cancers is primarily due the etiology: risk factor based or HPV induced [115]. The genetic variations at specific positions in the genome are considered to be common to all the subtypes of HNSCC with the intra- and inter-tumoral variations evolving with tumor progression. Classes of the tumor with high incidence of loss of heterozygosity (LOH) at specific regions of 3p and 9p are known to be associated with poor survival [116]. Comparative genomic hybridization (CGH) studies have also identified a subset of 20 % of HPV-negative HNSCC with near normal chromosomal/copy number variations [117, 118]. Expression profile-based differences have also identified distinct classes based on EGFR signatures, mesenchymal enrichment, epithelium type, and antioxidant enrichment that correlate to the survival and nodal metastasis [119–121]. HNSCCs are also reported to have distinct patient clusters with respect to the expression of the angiogenesis-related genes, indicating that the induction of angiogenesis probably occurs through distinct pathways [122].

The current model of progression in head and neck cancer is evolution through stepwise alterations in multiple molecular pathways [123]. The heterogeneity observed by the different studies in the head and neck tumors cannot be explained by this model alone. The cellular heterogeneity in the disease also indicates the presence of populations with stem cell characters along with multiple classes of differentiated cell types. These cell types have differential properties of tumor initiation, progression, and metastasis thereby suggesting multiple models of carcinogenesis which would probably account for the tumor heterogeneity.

13.3.1.2 The CSC Concept

The cancer stem cells (CSCs) constitute a small percentage of the total tumor cells and are considered the key to tumor initiation, metastases, and resistance to therapy in head and neck cancers, as is the case with the other solid tumors. Initial studies on the primary cultures of tissues from head and neck cancer patients provided evidence for the presence of a subgroup of cells with clonogenic properties. Cultures from HNSCC tissues showed a plating efficiency of 0.004–0.006 % in low-attachment experimental systems suggesting the presence of cells with anchorage independence. The efficiency correlated statistically with the stage of disease and decreased survival [124–127]. This subpopulation was first isolated by Prince et al., using CD44 as a marker and characterized to have properties of self-renewal, differentiation, clonogenicity, and tumorigenicity [128]. Subsequently, the side population (SP) of cells, known to represent cells with stem cell characters, has been isolated from several head and neck cancer cell lines [129–131]. *Asymmetrical cell division*, a distinct stem cell property [132], has also been attributed to this subset of cells. *Squamospheres*, considered to have an enriched population of stem cells, could be generated from primary head and neck cancers with all the specified characteristics [133, 134]. The presence of this subpopulation of cells with a higher tumorigenic and metastatic potential within the milieu of cells in head and neck cancer is no longer disputed; what needs elucidation is their specific role in tumorigenesis.

13.3.1.3 Origin of CSCs in Head and Neck Cancer

The origin of the CSCs or the "stemlike cancer cells" (SLCCs) as they are otherwise referred to has not been investigated thoroughly in HNSCC. A number of theories are in vogue with little evidence to support either of them with respect to head and neck cancer. The major concepts are (i) transformation of adult stem cells through a multistep molecular process and (ii) dedifferentiation of malignant tumor cells to generate the CSCs.

Transformation of Adult Stem Cells

Multiple lines of evidence are available to suggest that the stem cells are the target for mutations, at least in epithelial cancers. A prime argument for this concept is the fact that stem cells are the only subset of cells in the epithelial tissue that survive for a longer period of time, the average turnover of the epidermal cells being 6 weeks and that of the oral epithelium being 14 days [135, 136]. The long-standing adult

stem cells can thus accumulate the multiple genetic mutations necessary for transformation over a period of time. Though not much investigation has been carried out in head and neck cancers, an evidence for this concept in epithelial cancers comes in from carcinomas associated with the corneal epithelium. A majority of these carcinomas are known to occur in the peripheral limbal zone [137, 138] which is the prime site of the corneal epithelial stem cells [139, 140] suggesting a stem cell-based origin.

Dedifferentiation of Malignant Tumor Cells

Genomic instability is one of the hallmarks of most cancers; DNA damage inducers, UV rays, and mitomycin C are known to increase the stem cell population in nasopharyngeal cancers providing evidence for a possible transformation of cancer cells to cancer stem-like cells [141]. A similar effect was observed with an overexpression of key cell cycle regulators (*Mad2*) or knockdown of genes involved in mitosis (*Aurora B*) that are reported to increase genomic instability. The mechanism might differ from that observed during the transformation of tissue stem cells to CSC, but evidence does suggest the existence of this mode of origin in head and neck cancers.

HPV-Mediated Transformation

Human papillomavirus infects the basal layer of the epithelium and the infection follows the differentiation process of the epithelial cells [142]. The basal cell layer serves as a reservoir of stem cells which regenerates the epithelial layer by differentiation [143]. A micro injury to the epithelium generally activates these stem cells [143]; the same can also facilitate the entry of the virus. It is suggested that HPV, which is known to target proliferating cells, can infect these proliferating/activated "stem cells," transforming them to "cancer stem cell-like" cells [144, 145]. The virus remains latent in these cells, switching on its genetic machinery only when the stem cells divide and differentiate into proliferating suprabasal cells of the epithelium [146]. Evidences that correlate prognosis/aggressiveness of tumor with HPV positivity and the presence of the CSC markers further support this theory [147–149]. The other concept in vogue is that the virus directly infects the proliferating epithelial cells and initiates the carcinogenic process. Though the evidence in support of either of these theories is arguable, the location of HPV in the basal layer and the life cycle of infection do strongly suggest that CSCs can be generated via (Fig. 13.2) HPV-mediated transformation.

13.3.2 Cancer Stem Cells in Epithelial-Mesenchymal Transition and Metastases

Epithelial-mesenchymal transition (EMT) is a key event in epithelial cancer progression and metastases; during the process, cell-cell and cell-matrix interactions are altered, and the cytoskeleton is modified to enable navigation across tissues. During EMT under transformed conditions (EMT type 3), cells attain the capacity

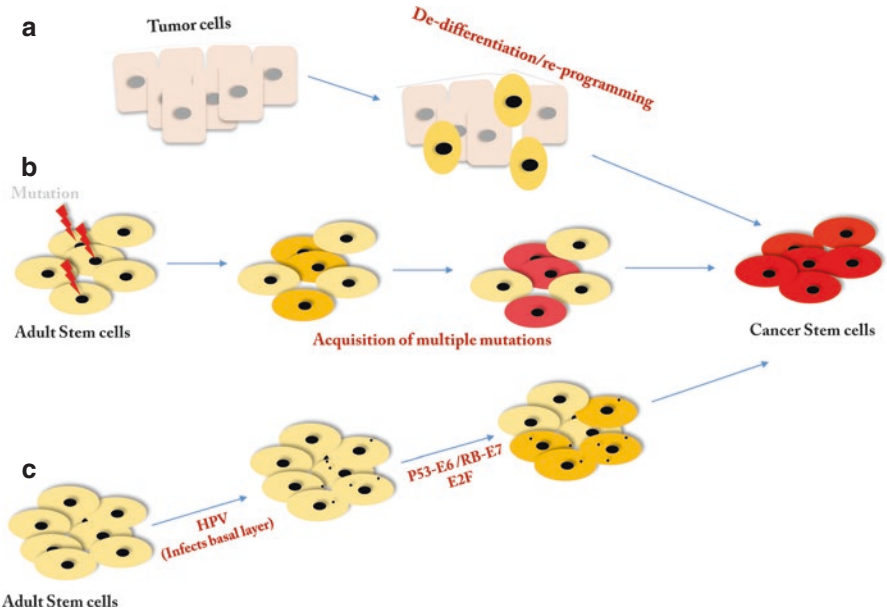

Fig. 13.2 Origin of cancer stem cells. (**a**) Dedifferentiation or reprogramming of the cancer cells through multiple pathways is one of the modes of CSC origin. The dedifferentiation process is said to be accomplished by activation of oncogenes and through the interaction with the niche components. (**b**) Transformation of tissue-specific adult stem cells by acquisition of multiple mutations due to carcinogenic insult is one of the accepted concepts of CSC origin. (**c**) HPV-mediated transformation of adult stem cells. HPV infects the basal layer of the epithelium in HNSCC patients, wherein the major cache of stem cells reside. The major pathways of HPV-mediated transformation: E6- and E7-dependent degradation of p53 and RB, respectively, are suggested to be associated with acquisition of CSC-like characteristics

to infiltrate surrounding tissue and metastasize to distant sites [150, 151]. These consequences obtained as a result are quite different as compared to the transitions (differentiation and regeneration) observed during embryonal development (EMT type 1) and during wound healing (EMT type 2) [151] (Fig. 13.3). The mesenchymal cells on reaching the destination undergo a reverse process, mesenchymal-epithelial transition (MET), and revert back to their epithelial phenotype. These cells, with metastatic potential, then proliferate to form the secondary tumor.

All the cells in the tumor do not undergo the process of EMT; in this regard, the idea that the cancer stem cells might be better equipped to be involved in the process gains significance [152]. Induction of EMT *in vitro* has been shown to induce the stem cell-like properties in mammary epithelial cells, while stem cells are known to express markers of EMT and can undergo the process [153]. Spheroid-derived cells from head and neck cancer cell lines show decreased *E-cadherin* (a hallmark of EMT) and an accompanying increase in the EMT-associated transcription factors such as *Snail* and *Twist* [154]. Conversely, downregulation of *Snail* inhibited the stemlike properties of the head and neck cancer stem cells [155]. An inducer of EMT,

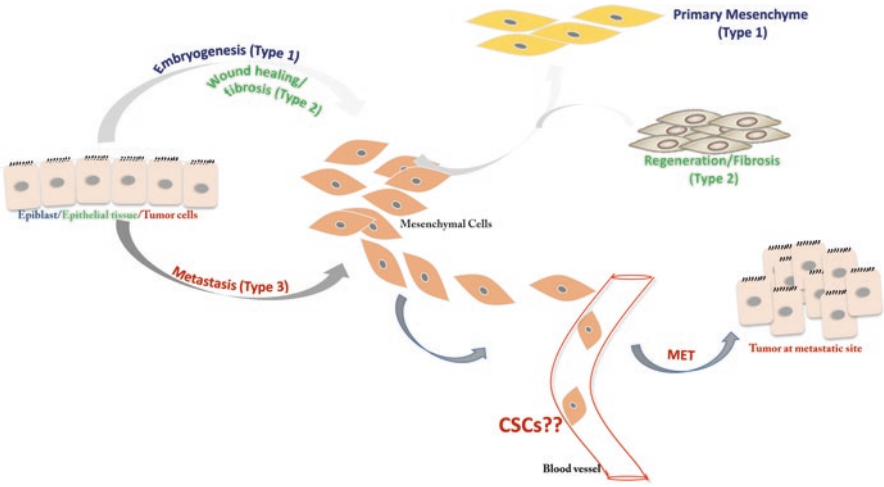

Fig. 13.3 Cancer stem cells and epithelial mesenchymal transition (EMT). EMT is involved in multiple processes under normal physiological conditions as well as in cancer. It is involved in embryogenesis during the development of the primary mesenchyme from the epiblast, ultimately leading to the formation of secondary epithelia (type 1). The second type of EMT (type 2) is involved in wound healing and regeneration leading to fibrosis and usually occurs following trauma and injury. Type 3 EMT occurs in neoplastic or cancer cells and provides for outcomes completely different from type 1 and type 2 EMT. Activation of EMT machinery in cancer cells provides for the cells to become more invasive, attain mesenchymal properties, circulate through the vascular system, and lead to distant metastasis in the patients. CSCs with their properties of tumorigenicity, increased migration, and invasion are suggested to be the cells that undergo this process of type 3 EMT resulting in the formation of metastatic tumors at distant sites

S100A4, is known to be essential in the maintenance of head and neck cancer-initiating cells [156]. The let 7 family of *mi*RNA generally functions as a tumor suppressor and a downregulation of its members is known to promote EMT in cancer stem cells [157, 158]. Further, several molecules (G8, AGR2) correlate with both EMT and CSC behavior in head and neck squamous cell carcinoma [159–161].

Evidence also points out to the select cancer stem cell population in HNSCC having a higher potential to metastasize as observed by *in vitro* and *in vivo* studies [162–164]. Cell lines derived from highly metastatic tumors, M3a2 and M4e, contained a higher percentage of side population cells as compared to the nonmetastatic cell lines [165, 166]. The evidence is not conclusive but does definitely suggests that the process of EMT, essential for metastasis in HNSCC, might well be a function of its constituent cancer stem cell population, the *metastatic stem cells*.

Clinically, a co-expression of *ALDH, Bmi1*, and *Snail* predicted a worst prognosis in head and neck cancer [167]. The expression of stem cell markers in HNSCC patients is also known to have a significant association with the development of recurrent tumors, metastasis, and survival [168]. The 5-year survival rates in cancers of the larynx, oropharynx, and hypopharynx are associated with overexpression of *CD44* in the tumors [169], while the frequency of CD44+Lin- cells and formation

of xenografts are much higher in the recurrent patients (36 % and 75 %, respectively) as compared to nonrecurrent patients (15 % and 21 %) [170]. These evidences suggest a definite involvement of the stem cells in the metastatic process and that their presence in the tumor does correlate to the metastatic behavior of the tumor and the clinical status of the patient.

13.3.3 Effect of the CSC Niche

The significance of the "seed-soil" hypothesis in the functioning of the normal tissue stem cells has been well established. The role of the niche, consisting of the stromal cells and the microenvironment, is highly under investigated in many cancers, especially head and neck cancer. Studies have shown that primary tongue tumors and paired metastatic lymph nodes do host cancer-associated fibroblasts (CAFs) [171]. Metastatic carcinoma cells in HNSCC are also known to downregulate E-cadherin and express other markers of EMT in the periphery of the cancer islands, wherein there is a direct contact with these cells of the microenvironment. These results suggest a role for the CAFs in tumor invasion and metastasis [171]. Studies have reported that 80 % of the CSC population (ALDH + CD44 + Lin-) identified in head and neck cancer is in close proximity to the blood vessels indicating the presence of a perivascular niche [172]. Endothelial secretary factors (EGF, Il-6) are also known to promote the self-renewal of CSCs accompanied with an increase in *Bmi1* expression and conversely, selective ablation of the tumor-associated endothelial cells induced a marked decrease in the CSC population in the xenografts [172–174]. Though it is understood that the niche does exert its effect in the carcinogenic potential of cancer stem cells in head and neck cancer [175], its exact role is yet to be thoroughly investigated.

13.3.4 Role in Therapy Resistance

The inherent and acquired resistances to therapy in HNSCC patients are prime deterrents toward achieving better survival rates and reducing morbidity. Cancer stem cells have been suggested to be responsible for therapy resistance in most solid tumors, primarily the acquired resistance. Studies in patients diagnosed with the disease showed that the response to therapy in patients with HNSCC has been reported to correlate with the lower expression of stem cell markers such as *c-Met* [176].

The side population cells isolated from HNSCC are known to be resistant to drugs such as 5-fluorouracil (5FU) [130] and to radiation [131]. Cells expressing standard stem cell markers such as *CD133, CD44, ALDH1*, and *c-Met* are resistant to chemotherapeutic drugs either due to the presence of drug efflux proteins (ABCG2) or due to resistance to apoptosis [155, 163, 177–179].

The presence of a dormant population of transformed stem cells and their subsequent enrichment after exposure to the chemotherapy are suggested to be the prime

reason for the resistant behavior of the tumors. Initial studies had reported an increase in tumorigenic potential in the small population of head and neck cancer cells that survived cisplatin treatment [180, 181] or treatment with drug combination [182]. These resistant sublines, enriched in CD44+ cells, also acquired characteristics of self-renewal, apoptosis escape, and migration [168]. Subsequently, this phenomenon was also documented in laryngeal carcinoma cell lines (Hep-2), wherein a major proportion of the cells that survived after exposure to drug treatment were CD133+ [177]; the proportion of CD133+ cells in the untreated cell lines were much lower in comparison.

13.3.5 CSC in Recurrent Disease and Second Primary Cancers

The concept of field cancerization suggests that the initial malignant transformation occurs in the stem cells, which subsequently forms a patch of altered cells and then expands into a larger field [183]. In the oral cavity, this altered field is known to be present in dimensions of over 7 cm in diameter. The residual cells in the field often remain after surgery and may lead to the formation of second primaries or local recurrence [184, 185]. The expression of *Bmi1* and Podoplanin (*PDPN*), markers of cancer stem cells, is known to be associated with recurrence and disease-free survival in oral cancer and esophageal cancer, respectively, suggesting the recurrent behavior to be a consequence of residual stem cells post treatment. In addition, expression of markers such as ATR1 and ABCG2 in the adjacent normal has also been associated with disease recurrence in tongue cancers [186]. *Skp 2* (S-phase kinase-associated protein 2) expression, important in stem cell maintenance, is also known to be significantly correlated with overall and disease-free survival in nasopharyngeal cancer [187, 188].

13.3.6 Molecular Profile of HNSCC CSCs

An understanding of the biology of the stemlike cells and establishing a clear molecular basis for their varied properties, self-renewal, therapy resistance, and metastatic potential will also enable designing of methods to investigate possible susceptibilities and devise new ways of targeting them. Studies down the years have catalogued the molecular profile of the cancer stem cells of HNSCC that can explain the properties attributed to them (Table 13.1).

13.3.6.1 Markers of Cancer Stem Cell Identification and Their Clinical Relevance

CD44

The expression of *CD44* correlates with the 5-year survival in HNSCC patients [169] with a higher of CD44+ cells identified in the peripheral blood of patients as compared to the controls [189]. The frequency of the CD44 cells in the

Table 13.1 Cancer stem cell markers in head and neck cancer

SL No	Marker	Functional attributes/clinical relevance of the cells	Reference
Markers for CSC identification and isolation			
1	CD44	Overall survival, disease recurrence, expression correlated with patients with risk habits, imparts higher motility	Faber et al. (2011) [189], Kokko et al. (2011) [169], Joshua et al. (2011) [170], Davis et al. (2010) [162], Mack and Gires (2008) [190]
2	ALDH1	Radioresistance, chemoresistance	Chen et al. (2009) [155], Chen et al. (2011) [154], Yu et al. (2011) [158]; Chen et al. (2009) [166]
3	CD133	Self-renewal, chemoresistance	Yang Jing-pu et al. (2011) [177]
4	IL-6	Tumorigenicity, increased levels post chemotherapy	Poth et al. (2010) [180]
5	PDPN	Tumorigenicity, asymmetrical cell division	Atsumi et al. (2008) [191]
6	*c*-Met	Self-renewal, chemoresistance	Sun and Wang (2011) [163]
7	OCT4	Associated with grade and differentiation of tumors	Chiou et al. (2008) [192], Yanamoto et al. (2011) [193], Lim et al. (2011) [133], Tsai et al. (2011) [181]
8	Nanog	Associated with grade and differentiation of tumors	Chiou et al. (2008) [192], Chen et al. (2011) [154], Tsai et al. (2011) [181]
9	EGFR	Activation enriches the SP cells	Chen et al. (2006) [194]
10	ALDH1+/CD44+/CD24+	High radioresistance and EMT	Chen et al. (2009) [155], Chen et al. (2011) [154]
11	Lin-CD44+	Correlation with recurrence and xenograft formation	Joshua et al. (2011) [170]
12	ALDH+/CD44+/Lin-	Highly tumorigenic, located in vicinity of blood vessels	Krishnamurthy et al. (2010) [172]
13	c-Met+/CD44+	High self-renewal, chemoresistance	Sun and Wang (2011) [163]
14	OCT4+/Nanog+/CD133+	Correlates with worst prognosis in patients	Chiou et al. (2008) [192]
Markers associated with treatment resistance			
1	ABCG2	Drug efflux, chemoresistance	Song et al. (2010) [165], Lim et al. (2011) [133], Yanamoto et al. (2011) [193]
2	ABCB1	Drug efflux, chemoresistance	Okamoto et al. (2009) [195]
3	ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCA2,	Chemoresistance	Yajima et al. (2009) [179]

(continued)

Table 13.1 (continued)

SL No	Marker	Functional attributes/clinical relevance of the cells	Reference
4	CFLAR, BCL2, BCL2A1	Resistant to chemotherapy-induced apoptosis	Yajima et al. (2010)
5	Bcl2, IAP	Resistant to apoptosis signaling	Chikamatsu et al. (2011) [178]
Markers associated with EMT and metastasis			
1	Wnt/β-catenin signaling	Correlated with the presence of metastatic lymph nodes	Song et al. (2010) [165]
2	Vimentin, cadherin, α-SMA	Spheroids show EMT (ALDH+/CD44+/CD24+)	Chen et al. (2011) [154]
3	miR200c	Low metastatic potential	Lo et al. (2011) [156, 196]
4	Bmi1	Enhances metastatic potential	Lo et al. (2011) [196], Yu et al. (2011) [158]; Chen et al. (2009) [155, 166]
5	S100A4	Cells show EMT	Lo et al. (2011) [156, 196]
6	BMP4	Cells show EMT	Qiao et al. (2011) [197]
7	MMP9	High invasiveness and metastasis	Sterz et al. (2010) [198]
Markers of stem cell maintenance and other markers			
1	Lrig1	Cells with low expression signify poor prognosis; Lrig negatively regulates EGFR; present in normal stem cells	Jensen et al. (2008) [199]
2	MAP4	CSCs show downregulation	Jensen et al. (2009) [200]
3	MSCP	CSCs show upregulation	Jensen et al. (2009) [200]
4	Nestin	CSC and NSC positive for marker	Chiou et al. (2008) [192]
5	CD117 (c-kit)	Cells show stem cell characters; overexpression leads to tumorigenicity	Chiou et al. (2008) [192]
6	CK18	Keratinocyte markers; negative in normal and cancer stem cells	Chiou et al. (2008) [192]
7	Notch 1 signaling/Hes	Stem cell maintenance	Zhang et al. (2010) [201]
8	Sox-2	Stem cell maintenance	Zhang et al. (2010) [201]
9	CK5	Expressed in squamospheres	Lim et al. (2011) [133]
10	CD29	Expressed in SP cells	Harper et al. (2007) [134]
11	EpCAM	Cells show CSC characters	Yanamoto et al. (2011) [193]
12	Snail/Twist	Associated with EMT	Lo et al. (2011) [196]

The molecular markers identified in head and neck cancer have been listed according to the functional relevance. The functions and clinical relevance of the cells positive for these markers are also listed

patients also correlated with the overall survival [202] and recurrence (36 %) (patients without recurrence 15 %) [170]. Cells positive for CD44 were one of the first subpopulations to be isolated from HNSCC with stemlike characters [128, 203–206]. The properties attributed to the CD44+ cells are active involvement in metastasis [162, 195], resistance to apoptosis due to expression of anti-apoptotic genes such as *Bcl-2* and inhibitor of apoptosis (*IAP*) [178]. CD44 marker expression in head and neck cancer is also known to be predictive of outcome to radiotherapy [207–209].

CD133

Studies in HNSCC have identified the cell surface marker *CD133* (Prominin 1) as an indicator of cells with stem cell-like properties [210]; up to 5 % of the cells are positive for the marker and exhibiting properties such as self-renewal, extensive potential for proliferation and differentiation, and in vivo tumorigenicity [210–213]. The presence of CD133 + or double positive cells with other CSC markers (ALDH1, CD44) has also been reported to be poor prognosticators [202, 214]. Further, the presence of these cells in the peripheral blood has also been indicative of distinct clinical implications [215].

ALDH1

Aldehyde dehydrogenase 1 (*ALDH1*) has also been investigated extensively, in isolation and in combination with *CD44* and *CD133*, for its potential as a stem cell marker in HNSCC. Cells positive for the markers exhibited extensive chemoresistance with properties of EMT [155]. ALDH+ cells also showed a downregulation of the *Let7 mi*RNA family of tumor suppressors, exogenous overexpression of which effectively blocked tumor metastasis [158]. Normal stem cells of the mammary gland are reported to express *ALDH1* along with their malignant counterparts [216], and investigations are required to see if this holds true in head and neck cancer as well. Presence of ALDH1A1+ cells in locally advanced, metastasized, head and neck cancers is suggested to indicate poor prognosis [217].

Other Markers

A number of other markers have also been reported to signify the stem cell population in HNSCC; *Oct-4* and *Nanog*, known to be responsible for normal stem cell maintenance, have also been extensively expressed in the CSCs (spheroids, SP cells) isolated from HNSCC and the Oct4+/Nanog + cells are known to be highly chemoresistant, with the expression correlating with the grades/differentiation of the tumors [133, 154, 181, 192, 193]. The association of *EGFR* with the maintenance of the stem cell population in the disease is not clearly established; activation of the receptor using the EGF ligand substantially increased the side population in the cell lines and conversely, its inhibition using *Iressa* decreased the population of cells [194]. Its role in the stem cell maintenance needs to be investigated, though its use as a stem cell marker is limited due to its established high expression in the non-stem tumor cells of HNSCC [218, 219].

Subset of populations from HNSCC expressing c-Met and Podoplanin (*PDPN*) exhibited SC characters and resistance to chemotherapeutic drugs [163, 191]. PDPN+ cells also showed asymmetrical cell division, a property of stem cells giving raise to both PDPN+ and PDPN- cells [191]. A comparison of the stem cell populations identified from cell lines before and after cisplatin treatment identified a transient increase in the expression of *IL-6* (interleukin-6) in the latter with the cells also showing a comparatively higher tumorigenic potential [180]. The maintenance of the stem cell phenotype in nasopharyngeal carcinoma (NPC) cell lines was affected by *Skp2,* a protein involved in cell cycle control and overexpressed in a variety of human cancers [187]. Recent reports also implicate other markers such as WNT5A [220], histone methyltransferase G9a [160], Hippo transducer TAZ [161], EMT-related transcription factor (ZEB1/ZEB2) [221], and CD44 v9 with acquisition and maintenance of cancer stem cell characters and also prognosis in head and neck squamous cell carcinoma.

Studies point out to the significance of the use of marker combinations for the identification/isolation of the cells with stemlike characters. In most of the studies, stem cells identified with marker combinations (double positive/triple positive) showed a higher tumorigenic potential and clonogenic properties as opposed to their counterparts identified with single markers. ALDH1+/CD44+/CD24+ and ALDH+/CD44+lin- cells showed high tumorigenicity, increased radioresistance, and propensity for EMT as compared to ALDH+ or CD44+ cells [155]. Similar results were observed in studies wherein the combinations of *c*-Met and CD44 [163] or Oct4/Nanog and CD133 were used [192]. This strongly suggests that there is a definite increase in the specificity of the cells isolated on the use of multiple marker combinations.

13.3.6.2 Markers Associated with Treatment Resistance

ABC Family

Gene expression profiling of the side population (SP) of cells isolated from the head and neck cancer cell lines showed a differential profile for the ABC family genes: *ABCB1* and *ABCG2* in the SP as compared to the non-SP cells [130, 179]. The higher chemoresistance observed in the side population cells, CD44+ and/or CD133+ cells, correlated to the expression of *ABCG2* in a majority of the studies [132, 165, 177, 195]. Squamospheres derived from HNSCC, which are resistant to most of the drugs currently in use (cisplatin, 5-fluorouracil (FU), paclitaxel, and docetaxel), have high levels of *ABCG2* as compared to the other tumor cells [133]. Other members of the family, *ABCC1, ABCC2, ABCC3, ABCC4, ABCC5*, and *ABCA2*, have also been implicated in chemoresistance [179].

Anti-apoptotic Markers

Expression of anti-apoptotic markers is an established mechanism of acquiring resistance to therapy. The genes *CFLAR*, *BCL2*, and *BCL2A1* are upregulated in the SP cells of the head and neck cancer cell lines [179]. Resistance to apoptosis signaling has also been identified in CD44-enriched cells of chemoresistant cell lines

[168, 182]; while CD44+ cells isolated from head and neck cancer tissues showed overexpression of Bcl-2 and IAP [178].

13.3.6.3 Markers Associated with Epithelial-Mesenchymal Transition and Metastasis

Markers of EMT, such as *Bmi1* (B lymphoma Mo-MLV insertion region 1 homolog), the polycomb ring finger oncogene, *Snail* (SNAI1), and *Twist* [222–230], are known to regulate the metastatic potential of the cancer stem cells in HNSCC. A knockdown of the *Snail/Bmi1* mRNA in the *CD44+/ALDH1+* positive cells resulted in blockage of their tumorigenic and metastatic properties with an increased sensitization to radiation treatment [155, 167]. *Bmi1* is also known to be upregulated in the cells double positive for *CD44* and *CD133* and with higher tumorigenic and metastatic potential [195, 231]. Side populations from metastatic HNSCC cell lines showed an upregulation of Wnt/β-catenin signaling accompanied by expression of *Bmi1* and other stem cell markers (*CD24, CD44*) [165]. ALDH+ cells derived from spheroid cultures with high invading and metastatic capability, showed the expression of known EMT markers such as *Vimentin* and *α-SMA* and a downregulation of *E-cadherin* [232]. These markers are also known to be poor prognosticators in patients with head and neck cancer [79, 233].

Among the *mi*RNA markers, *mi*R200c is downregulated in the ALDH+/CD44+ cells and an overexpression inhibited the malignant potential of the cells of the oral cancer cells and reduced the expression of genes involved in EMT (*ZEB1, Snail, N-cadherin*) [196]. A comparative increase in Bmi1 is observed in these cells with low *mi*R200c [196]. Conversely, cells positive for *S100A4*, an inducer of EMT, have properties of self-renewal with the overexpression correlating to the stem cell properties, grading, and survival in the patients with HNSCC [156]. *mi*R300 is reported to regulate EMT through the downregulation of TWIST [234], while the TGFB-miR200 axis is known to regulate EMT and thereby provide resistance to anti-EGFR-targeted therapy [235]. *BMP4* (bone morphogenetic protein-4), a known inducer of EMT in ovarian cancer, is also shown to introduce changes in the morphology of the isolated cancer stem cell population and increase expression of *ABCG2* and markers of EMT [197] in oral cancer. Matrix metalloproteinase 9 (*MMP 9*) is a known marker for invasiveness and metastasis; comparison with *CD44* expression has shown their co-localization at the invasive front in HNSCC tumor specimens [198] with a significant correlation to the invasive properties of the tumor.

13.3.6.4 Molecular Profile in Common with the Normal Oral Stem Cells

The high capacity of regeneration observed in the oral mucosa warrants the presence of a substantial population of normal stem cells in the epithelium. A majority of these cells are located in the oral mucosal *lamina propia* (OMLP). These cells that form a cord in the OMLP are known to be positive for the transcription factors, *Oct4*, *Nanog*, and *Sox2*. Analysis has shown that 95 % of these cells express mesenchymal stromal cell markers, with about 40–60 % expressing the SC transcription factors. These cells also showed the propensity to generate tumors on treatment with

dexamethasone, suggesting that they can be transformed into their tumorigenic counterparts [236]. Oral keratinocyte stem cells showed primarily the expression of markers such as *CD71*, $\alpha_6\beta_4$ integrin with the cells also being positive for *Oct3/4, CD44H*, and *CK19* [237]. *CD44*, a marker that has been used to identify CSCs from HNSCC, has also been identified by other studies as expressed in the normal epithelial tissue [190]. The distinct similarities observed between the normal and the cancer stem cells in terms of their properties and marker profile make it very difficult to discriminate between them, an aspect which needs to be thoroughly looked into when developing cancer stem cell-based targeted therapy.

13.4 Cancer Stem Cells and Therapy

Therapeutic strategies applied in cancers have advanced manyfold down the decade; nevertheless, the increasing rates of recurrence and development of secondary tumors, after an initial response to therapy, are a constant concern. Evidences accumulated in recent years point out to the concept that in a majority of the cases, carcinogenesis and malignant transformation are consequences of the deregulation of the normal process of differentiation. The tumorigenic potential in these cases is believed to originate from an aberration in a subgroup of cells, within the tumor, with characteristics that differ from the majority of tumor cell population. This concept provides an understanding for the increasing rates of treatment failure, since most of the current modalities target the latter, i.e., the majority tumor cell population.

13.4.1 Current Therapy and CSCs

The anticancer therapeutic modules currently adopted are all targeted against the rapidly dividing tumor cells that form the bulk of a tumor. Extensive studies in a variety of cancers and in head and neck cancer have identified that the subgroup of slow-dividing cells with stem cell characteristics within the tumor are largely resistant to the cytotoxic effects of radiation and the chemotherapy. Furthermore, evidence also points out that exposure of these cells to these treatment modalities leads to an enrichment of this population, a highly malignant and aggressive recurrent tumor being the consequence.

The use of therapy in tumors is widely known to reduce the tumor volume but does not provide local control, an observation that can be clearly explained by the resistant behavior of the resident cancer stem cells in the tumor [238]. As discussed previously, molecular studies have revealed a correlation between the prognosis/survival of the patient with the marker profile specifying this subgroup of cells. The presence of stem cell markers such as *CD44*, *CD133*, and *cMET* has been predictive of resistance to radiation and chemotherapy in a number of tumors [163, 207, 208, 239], with the frequency of recurrence being higher in patients with highest expression [170].

As a next line of evidence, the cancer stem cells isolated from the tumor samples of different sites have also been shown to be resistant to the currently used drugs such as cisplatin, paclitaxel, temozolomide, carboplatin, paclitaxel (Taxol), bortezomib, and etoposide (VP16) [163, 165, 181, 201, 239]. An exposure to these drugs was also observed to enhance the proportion of the cancer stem cells in the tumor [181]. Studies in breast cancer have shown an increase in the CD44+/CD24− population subsequent to therapy. This subpopulation of cells was decreased on use of *lapatinib* (inhibitor of EGFR/HER2 pathway), but this decrease did not correlate with the long-term clinical outcome; reasons may either be an incomplete elimination of the CSC population or the presence of a resistant CSC niche [240].

Resistance to radiation has also been documented in a large number of tumors: CD133+ cells in glioblastoma and ALDH+/CD44+ and CD44+/CD24− cells in breast cancer [99, 241, 242]. In glioblastoma, analysis of the CD133+ cells indicated that they differ from their CD133− counterparts in the presence of an active DNA damage repair pathway and less apoptosis following radiation by inducing the checkpoint kinases [99].

The effect of the niche on the origin and metastatic potential of cancer stem cells is an accepted concept; consequently, an effect on the therapeutic potential is also expected. An evidence for this "effect" was observed in CD34+CD38-CD123+ leukemic stem and progenitor cells (LSPC) treated with *cytosine arabinoside* (Ara-C) and with the *FLT3* inhibitor AG1296. The cells were sensitive to the drugs when cultured without microenvironmental support, but in the presence of niche-like conditions, their survival/resistance was enhanced [243].

The resistant property of the cancer stem cell population in the tumor, which forms a minority cell type, makes it imperative to adopt novel approaches that target these cells. The therapeutic modules currently being investigated thus focus on strategies that target this cell population and its niche.

13.4.2 Targeting CSCs and Its Niche

13.4.2.1 Major Strategies, Targets, and Modulators for CSC: Therapy in Cancers

Research in the past decade has identified candidate markers that specify the cancer stem cell population and also evaluated their potential as therapeutic targets. Considering the heterogeneity of the tumor population, it would also be imperative to adopt combination therapies that target independent pathways to attain near complete elimination. The potential approaches suggested for targeting CSCs would be (i) CSC-specific therapy, (ii) anti-SC therapy (effects CSCs and NSCs), and (iii) combination therapy (Fig. 13.4) [244]. Differentiation and elimination therapy wherein the CSCs either are induced to differentiate using multiple mechanisms such as epigenetic modulations [245] or are eliminated themselves, respectively [246], have been explored in a number of cancers using molecular markers implicated in CSC maintenance and tumorigenicity.

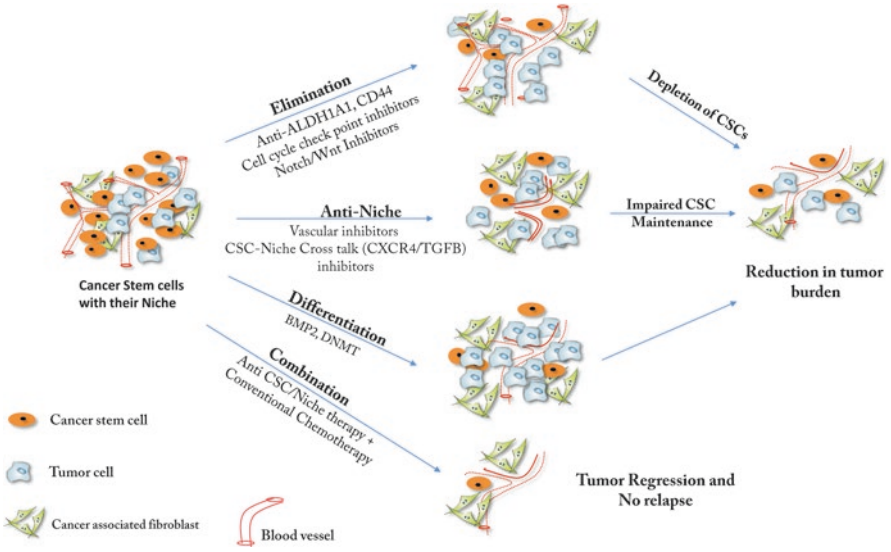

Fig. 13.4 Targeting the cancer stem cells. CSC targeting includes multiple approaches that can be adopted. Elimination therapy includes specific targeting using CSC-specific strategies (anti-ALDH1A1, CD44, cell cycle checkpoint inhibitors) that can deplete the CSC cache in the tumor. Targeting the vascular/fibroblast niche and the cross talk between the CSC-niche can lead to an inhibition of the cross talk thereby impairing the CSC maintenance. Differentiation therapy, on the other hand, includes using strategies (BMP2, DNMT) towards differentiating the CSCs into cancer cells and thereby reducing the overall tumor burden. Combination therapy including anti-CSC targeting along with the conventional/standard chemotherapy (Platinum, Taxol, 5FU) will probably be the best approach, leading to simultaneous depletion of CSCs and a reduction in the overall tumor burden. This can also be an approach that can prevent disease relapse at a later stage

Differentiation Therapy

Modulators that can induce differentiation in the CSC are used in the differentiation therapy approach; BMPs, soluble factor that can induce differentiation of neural precursors, can also induce CD133+ cells to differentiate, thereby reducing their tumor-initiating capacity and rendering them sensitive to therapy [247]. *BMP2* is also known to induce differentiation of human pluripotent teratocarcinoma cells, thereby reducing their stem cell characteristics [248] while this approach has also been adopted in leukemia [249]. Targeting DNA methyltransferases (DNMT) is known to have differentiation-inducing effects in the CSC or the progenitors of leukemia, GBM, and other cancers [245].

Elimination Therapy

This approach involves selective targeting and subsequent elimination of the cancer stem cells using markers specific to these cells. The subset of markers that have been identified down the years have been explored for their therapeutic potential.

Targeting *ALDH1*, a CSC marker identified in a number of cancers, by inhibiting its activity using all-trans retinoic acid (ATRA) or the specific ALDH inhibitor diethylaminobenzaldehyde (DEAB) sensitized the ALDH+CD44+ population to doxorubicin/paclitaxel and/or radiotherapy in breast cancer cell lines [241]. Inhibition of *CD133* and *MDR1* using appropriate *si*RNA significantly reduced the sensitivity to paclitaxel in colon cancer stem cells [250]. The self-renewal properties of CD133+ cells were also successfully inhibited by blocking the cell cycle checkpoint proteins *CHK1* and *CHK2* [247] and by new-generation taxoid (SB-T-1214) in colorectal cancer [251]. Recent study has reported the success of targeting the c-Met/FZD8 axis in eliminating the CSC-like cells in HNSCC [252]. Other therapies such as metformin-dependent activation of FOX3 in GBM, CD123-CD3 targeting in leukemia, and SIRT1 inhibition in CML have been successful in eliminating corresponding tissue-specific CSCs [253–255].

Monoclonal antibodies against a number of the candidate CSC markers have been explored for their efficacy in treating tumors. Anti-CD44 antibodies have been the major approach; H90, A3D8, and other antibodies have been tested against AML, melanoma, and pancreatic cancer [256–260]. Antibodies against other tumor-associated antigens such as *CD133, ALDH1, EpCAM*, drug transporters (*ABCG2*), CD20, and Notch ligands have been effective in vitro and/or in vivo models [261–269]. A similar approach has been adopted against CSC-supporting factors such as *IL4, PSCA,* and *IL8* and the niche factors such as angiogenesis (*VEGF*), *CD24*, integrins, and chemokines [270–282] (Table 13.2).

Synergy between the *Notch* and *Wnt* pathway is instrumental in inhibiting terminal differentiation of neuronal stem cells and cancer stem cells. Targeting this pathway through its enhancer, Gl-1, by using inhibitory RNA, decreased the pool of CD44 (high) and CD24 (low) cells, suggesting that this might be effective in eliminating the taxol-resistant CSC population in ovarian cancer cell lines [285]. The PTEN pathway is implicated in a number of cancers; rapamycin, an inhibitor of PI3/AKT pathway, is known to be effective as a treatment strategy [286]. Inhibition of anti-apoptotic pathways has been effective in leukemias, and small molecule inhibitors that block the cytoprotective activity of the *Bcl-2* family have been tested in both in vitro and in vivo experimental models [287].

The use of activated cytotoxic T lymphocytes (CTLs) has also been employed in CSC targeting. Breast cancer CSCs (CD44+CD24 low) were eliminated by CTLs activated by peptides of *Numb-1*, a protein that is known to inhibit/degrade the proto-oncogene *Notch 1* [288].

Telomerase activity is essential in tumorigenic cells to prevent the shortening of the chromosome lengths during successive cell divisions; targeting the enzyme using GRN163L, a direct inhibitor has been effective in chronic lymphocytic leukemia, multiple myeloma, solid tumors, and non-small cell lung cancer. Considering the significance of telomerase in CSC maintenance, this inhibitor might play an important role in eliminating CSCs [289, 290].

A number of candidate markers are yet to be investigated for their potential in therapy. Leukemic stem cells show a high expression of *CD32* and *CD25,* markers

Table 13.2 Antibody-mediated anti-CSC therapies

Marker	Site	Antibody	Reference
CD44	Acute myeloid leukemia (AML)	H90, A3D8	Jin et al. (2006) [256]
	Pancreatic cancer	HuARH460-16-2	Young (2007) [260]
	Head and neck cancer	Bivatuzumab (BIWA-4)	Verel et al. (2002) [283]
EpCAM epidermal surface antigen	Colon, prostate	ING1, adecatumumab	Ammons et al. (2003) [264]
CD9	AML	AR40A746.2.1	Murayama et al. (2008) [281]
CD133, Prominin 1	Hepatocellular cancer	AC133, AC141	Smith et al. (2008) [261]
CD24 heat-stable antigen	Colon, pancreas	Anti-CD24 mAb	Sagiv et al. (2008) [271]
CXCR4	Multiple melanoma, prostate, colon	Anti-CXCR4 mAb	Muller et al. (2001) [280]
PSCA	Prostate	Hu2B3	Olafsen et al. (2007) [272]
DLL4	Colon, breast	21M18	D'Souza et al. (2008) [266]
Frizzled	Colon, breast	23M2, 44M13	Gurney (2012) [284]
Wnt	NSCLC	Anti-Wnt 1	He et al. (2004) [267]
Notch	Breast	90R21, 90R22, 90R29	Gurney (2012) [284]
Patched	Pancreas	Anti-patched mAb	Nakamura et al. (2007) [268]
Integrin	Prostate, colon	LM 609	Huveneers et al. (2007) [282]
VEGF/VEGFR	Glioma	Bevacizumab	Bao et al. (2006) [279]
CD20	Melanoma	Rituximab	Schlaak et al. (2012) [269]
ABCB5	Leukemia, melanoma	Anti-ABCB5 Ab	Schatton et al. (2008) [265]

which are not expressed in the normal hematopoietic lineage, indicating them to be potentially safe therapeutic targets [291]. The *STAT3* and HH/PTCH pathway have been implicated in CSCs of many cancers. Targeting the HH pathway using specific inhibitors such as cyclopamine has been reported to reduce tumorigenicity of prostate cancer cell lines; effectiveness against CSCs needs to be specifically investigated [292].

Besides the molecular targets mentioned afore, physiological targets such as reactive oxygen levels (ROS) and tumor vasculature may also prove effective against CSCs. Superoxide dismutases dependent on bivalent cations play an important role in neutralizing the more active forms of ROS; thus, the removal of some bivalent cations kills cancer cells [293]. The cellular redox state is known to influence the

self-renewal and differentiation potential of CSCs, and targeting the same might hence prove effective [247].

Combination Therapy

Approaches that include the combination of sensitizing and debulking agents along with single agents that target the CSC are suggested to be more effective in tumor regression. Studies have shown that treatment of CD133+ colon cancer cells with an antibody against IL-4 prior to treatment with oxaliplatin and 5-FU increased cell death [270]. Use of multiple agents targeting the different pathways may lead to a near complete elimination of the CSCs in the tumor.

13.4.2.2 Targeting the Niche

Though the therapeutic potential of CSC niche is largely unexplored, recent evidence does suggest that targeting the niche along with resident CSCs and/or molecules involved in cross talk could be a promising treatment strategy. The vascular niche is essential for CSC maintenance; strategies to isolate the endothelial cells from the CSCs by injecting molecules such as *IFN-β* that increased the intermediary perivascular cell component have been successful in reducing the tumor in gliomas [294]. The use of viral vectors has also been explored wherein vectors carrying fusion genes targeting the vascular niche could inhibit the proliferation of human brain microvascular endothelial cells [295]. Combining angiogenesis inhibitor with other targeted inhibitors is also suggested to be more effective since the tumor vasculature that nourishes the tumor will be terminated [296]. The major pathways involved in tumor-niche interactions such as TGF-B, Notch-1-DLL, and SDF-1/CXCR4 [297–299] are currently being explored for their role in the interactions of the tumor-initiating cells with the various niche components [300–302] and need to be further investigated as potential targets for single or combination therapy.

Some of the other extracellular matrix (ECM) molecules that have been targeted include fibronectin and hyaluronic acid. Antibodies against fibronectin receptor (*VLA-4*) inhibited the association of the tumor cells with the premetastatic niches, thereby reducing the incidence of residual disease in an acute myeloid leukemia model [303, 304]. Hyaluronic acid protects the HSCs from 5-U cytotoxicity and blocking its receptors reduced minimal residual disease in AML models [305].

The use of CSC-directed therapies also necessitates a re-evaluation of the methods used to evaluate their efficacy. The traditional end points such as initial reduction in tumor volume or burden may not hold true for anti-CSC therapies, which may not necessarily act in this manner. Contrary to the conventional therapies, longer treatment periods may be required to exhaust the resident CSCs and observe clinical responses. The primary end points probably need to be progression-free survival while secondary end points could be measurement of the tumor burden or ex vivo functional assays using isolated CSCs; clinical trials need to be designed to identify these end points for the different therapeutic strategies.

13.4.3 Anti-CSC Therapy and the NSC Population

The cancer stem cells share a majority of their characteristics with the resident normal stem cell population. Properties of self-renewal, proliferative potential, and drug resistance are common between the two cell types; consequently, one of the major adverse affects of anti-CSC therapy will be the effect on the normal stem cells (NSCs). Considering the fact that in tissues such as the oral mucosa, cells have a turnover period of 2 weeks; the NSCs play a vital role in normal maintenance and differentiation of the tissue. The selection of stem cell pathways as potential therapeutic targets thus needs to be carried out with extreme caution.

13.4.4 Prospects of CSC-Based Targeted Therapy in Head and Neck Cancer

13.4.4.1 Possible Candidates and Anti-CSC Therapy in HNSCC

Investigations with regard to the therapeutic possibilities against cancer stem cells in HNSCC are in the nascent stage; nevertheless, a few studies have explored the therapeutic potential of CSCs. Attempts to target the CD133 (+) cells have been carried out by conjugating the anti-CD133 antibody with the genetically modified cytolethal distending toxin (Cdt), from the periodontal pathogen *Aggregatibacter actinomycetemcomitans*. The Cdt-MAb complex preferentially inhibited the proliferation of CD133 (+) cells in cultures of established cell lines derived from HNSCC, with the inhibition being rate and dose dependent. The healthy primary gingival epithelial cells that are native targets of the wild-type Cdt were not affected [306]. Anti-CD44 monoclonal antibody (bivatuzumab) has also been developed for treatment against HNSCC [283]. Inhibition of Grp78, another SC marker in HNSCC cells, induced the pro-apoptotic pathway, thereby leading to depletion of the CSC population [307].

Cancer stem cells [CD44 (+) ALDH (+)] from HNSCC were also effectively inhibited *in vitro* by compounds such as Cucurbitacin I, with decrease in their stemness and radioresistance. Xenotransplant experiments revealed that Cucurbitacin I combined with radiotherapy significantly suppressed tumorigenesis and lung metastasis and further improved the survival rate in HNSCC-CD44 (+) ALDH1 (+)-transplanted immunocompromised mice [308]. In vivo/in vitro studies have also revealed inhibition of CSC targeting pathways/molecules such as mTOR pathway [309], cMET, and Wnt [252, 310].

*mi*RNA such as *mi*R200c and Let 7a negatively modulate BMI/Nanog expression thereby inhibiting tumorigenic and metastatic properties of CSCs in HNSCC. A restoration of miR 200c/Let 7a is suggested as a novel therapeutic approach to target CSCs specifically [196].

13.4.4.2 Candidates for Niche Targeting in HNSCC

An understanding of the CSC niche in HNSCC is in the preliminary stage; nevertheless, as in other tumors, targeting the niche will definitely be an approach to target the CSCs in HNSCC. The CSCs in HNSCC are known to be located in close

proximity to the blood vessels, and targeting the vascular niche can be adopted to eliminate the CSCs [172, 311]. The process of oral tumorigenesis is also assisted by other microenvironment-related factors such as hypoxia [312], while interactions between the tumor cells and fibroblast through molecules such as extracellular matrix metalloprotease inducer (EMMPRIN) and SDF-1/CXCR4 are known to induce a favorable environment for tumor growth [313]. Targeting the cancer stem cell niche using specific markers will hence be a beneficial approach towards eliminating the CSC population in the HNSCC tumors.

13.4.4.3 Other Available Modulators and Future Prospects

A variety of biomodulators have been tested against the CSC population of different tumors, and they can be evaluated in HNSCC as prospective candidates. P-glycoprotein is known to be overexpressed in a number of chemoresistant cancers and is also a known marker in HNSCC [314]. MMPT (5-[(4-methylphenyl) methylene]-2-(phenylamino)-4(5H)-thiazolone) may induce tumor-selective cell killing in both P-glycoprotein-negative and P-glycoprotein-positive cancer cells and could be a new anticancer agent for treatment of refractory tumors [315]. *Irofulven*, an alkylating agent, has been reported to have extensive antitumor activity against a number of chemoresistant ovarian cancer cell lines and also inhibit colony formation in surgically derived tumors, a characteristic of the inherent CSC population. The drug and its clinical relevance need to be further investigated in other cancers such as HNSCC [316].

Disulfiram, an anti-alcoholism drug, is known to inhibit proteasomes when complexed with different metals. Mammosphere formation and the ALDH1(+VE) and CD24(low)/CD44(high) CSC population in mammospheres were significantly inhibited by exposure to DS/Cu; the drug also induced reactive oxygen species (ROS) generation and activated apoptosis-related downstream pathways such as cJun N-terminal kinase and *p38 MAPK* while inhibiting the constitutive NFkappaB activity in breast cancer cell lines [317]. ALDH1 is established as the marker for HNSCC stem cells; effect on this drug in HNSCC remains to be investigated. Therapeutic approaches adopted (antibody mediated or others) in other cancers against other markers associated with CSCs/niche of HNSCC such as *CD133, IL6, ABCG2, Wnt, Notch1* CXCR4, NOTCH, and *EpCAM* need to be investigated in HNSCC. Anti-apoptotic therapies which have also shown to be effective in other cancers also need to be explored in head and neck cancer.

The application of anti-CSC-mediated therapy against head and neck cancer is hence an extremely viable approach to tackle the issues currently affecting the overall and disease-free survival rates in the disease. Research carried out down the years has identified a number of prospective candidates with therapeutic potential, with biomodulators against a subset of them being in the investigational stages in HNSCC and other cancers. Therapies which target pathways specific to the disease will probably be much more beneficial as compared to tumor-type approaches; considering the complexity of the process of carcinogenesis and the pathways involved, combination therapy would probably be the best option. The current focus of research in the field thus includes exploration studies to identify novel, functionally

relevant candidates specific to the CSC population and drug trials to evaluate the efficacy of the currently available modulators in HNSCC/other cancers and to develop new therapeutic strategies/approaches.

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1. Patel K., Kadamani D., Kuriakose M. A. (2017) Oral Potentially Malignant Disorders. In: Kuriakose M. (eds) Contemporary Oral Oncology. Springer, Cham

2. Oral cancer: leukoplakia and squamous cell carcinoma, Nelson L.Rhodus https://www.sciencedirect.com/science/article/abs/pii/S0011853204000886?via%3Dihub (2005, 2014)

3. Proliferative Verrucous Leukoplakia Treatment & Management, Rahat S Azfar https://emedicine.medscape.com/article/1081559-treatment (1994-2019)

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1. Priya R. Prabhu, M. Radhakrishna Pillai (2016) Human Papillomaviruses and Squamous Cell Carcinomas of Head and Neck Region. In: Kuriakose M. (eds) Contemporary Oral Oncology. Springer, Cham

2. Current trends in the etiology and diagnosis of HPV-related head and neck cancers, Ryan C Chai, Duncan Lambie, Mukesh Verma, and Chamindie Punyadeera, https://onlinelibrary.wiley.com/doi/full/10.1002/cam4.424. (2015)

3. Oral HPV Related Diseases: A Review and an Update, Helena Lucia Barroso dos Reis, Silvia Paula de Oliveira, Danielle Resende Camisasca, Juliana Bittencourt Pessoa, Ana Beatriz Azevedo Queiroz, Mauro Romero Leal Passos, Philippe Godefroy, Dennis de Carvalho Ferreira, Thais Camporez Pimentel and Antonio Chambo Filho, https://www.intechopen.com/books/trends-in-infectious-diseases/oralhpv-related-diseases-a-review-and-an-update (2014)

4. Detection of human papillomavirus (HPV) in clinical samples: Evolving methods and strategies for the accurate determination of HPV status of head and neck carcinomas, William H.Westra, https://www.sciencedirect.com/science/article/abs/pii/S1368837514001420?via%3Dih ub (2014)

5. HPV and cancer of the oral cavity, Christian U Hübbers & Baki Akgül, https://www.tandfonline.com/doi/full/10.1080/21505594.2014.999570 (2015) three paragraphs in beginning.

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Camile S. Farah, Nirav Bhatia, Yastira Lalla, An Vu, Keziah John, Vishal Gupta, John Baeten, Alexander Johnson, and Deepak Kademani

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