Chapter 3
Oral Carcinogenesis and Malignant Transformation

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Introduction

Cancer of the oral cavity and oropharynx is a significant health burden, with over 300,000 new cases diagnosed annually [1]. Oral squamous cell carcinoma (OSCC) constitutes 95% of these malignancies and is mostly preceded by lesions termed oral potentially malignant disorders (OPMDs) that have a high tendency for malignant transformation [2]. Despite advances in diagnosis and treatment modalities, the survival rate of OSCC has not changed significantly in the last five decades [3]. The poor prognosis of oral cancer can largely be attributed to its frequent diagnosis at an advanced stage [4]. Understanding the process and natural history of oral carcinogenesis has the capacity to improve the clinical outcomes of patients with OSCC through early detection and effective OPMD management. This chapter discusses the most recent concepts and knowledge on oral carcinogenesis and malignant transformation of OPMDs.

Oral Mucosa Development and Epithelial Differentiation

Given that OSCC arises from oral epithelium, understanding the normal anatomy, histology, biology and physiology of normal oral epithelial cells is a prerequisite to understanding oral carcinogenesis.

Oral mucosa lines the structures of the oral cavity and developmentally originates from ectoderm and ectomesenchyme—in particular, neural crest cells [5]. Given the sophisticated functionality of the oral mucosa, it has typically been

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subdivided into three categories: lining mucosa, masticatory mucosa and specialised mucosa. Despite this, the mucosa at all oral sites comprises a lining of stratified squamous epithelium (SSE), keratinised or non-keratinised, depending on the region and underlying connective tissue of the lamina propria and submucosa [6, 7].

Lining mucosa covers the lips, cheeks, soft palate, alveolar mucosa, and the floor of the mouth and is typically lined by stratified non-keratinised squamous epithelium. Surface cells are shed continuously and replaced by progenitor stem cells at the basal cell layer. The lamina propria has a similar pattern to that found in the dermis of the skin [6]. On the other hand, masticatory mucosa lines the hard palate and gingiva, while specialised mucosa is unique to the dorsum of the tongue. In both types of mucosa, masticatory and specialised, the lining epithelium is stratified keratinised squamous. They both have elongated rete ridges and relatively dense lamina propria to reinforce the cohesion between epithelium and lamina propria [6, 7].

The oral epithelium has four layers as a result of cell proliferation and sequential differentiation: stratum germinativum (or stratum basale), stratum spinosum (or prickle cell layer), stratum granulosum (or granular layer) and stratum corneum (the keratinised or cornified layer) [6, 7]. The oral epithelium has two cell populations: progenitor (cells are responsible for dividing and providing new cells) and maturing (cells undergo continuing process of differentiation to form a protective barrier) [7]. The final pathway of SSE differentiation allows either reaching entirely cornified dead cells (squames) that are found on the hard palate and gingiva or non-cornified as is seen in lining mucosa. Interestingly, the cytoskeleton of oral epithelial cells has individual intermediate filaments known as cytokeratins (CK) which are products of the CK genes. Cytokeratins are a group of at least 20 subtypes [7]. The phenotype of the cytokeratin reflects the differentiation pathway of the epithelial cells [7]. For example, CK 14 is strongly positive in the basal cell layer. It is important to know that the CK profile changes in the oral epithelium with pathological situations [6, 7].

Embryonic development mostly dictates the distribution of keratinisation in the oral epithelium [8]; however, there are some normal variations seen in adults. In fact, normal proliferation and differentiation of oral epithelium are controlled by autocrine and paracrine factors that are generated by keratinocytes, cytokines, growth factors and circulating systemic factors [6]. Another influencing factor is due to epithelial/mesenchymal interaction [6]. Some researchers argue that mesenchyme plays a significant role in determining the phenotype and morphology of the overlying epithelium. Additionally, gap junctional communication is documented to have a role in regulating oral epithelia differentiation [6]. Moreover, local stimuli in the oral cavity can induce reversible changes depending on the persistence of the stimuli [7]. For example, chronic physical irritation may cause hyperkeratosis of the buccal mucosa. Additionally, inflammation may affect the mitotic activity of oral epithelial cells. Mild subepithelial inflammatory infiltrate may stimulate proliferative activity of the oral progenitor cells, while severe inflammation may cause marked reduction in this activity. These effects probably represent the influence of cytokines [7].

The balance between cell loss and cell formation has a delicate role in stabilising the homeostasis of multilayered oral squamous epithelium [8, 9]. Oral epithelial cells normally divide at different rates, and it is possible to estimate the time necessary to replace all the cells in the epithelium, known as turnover time [8].

Importantly, the oral epithelium divides following a unique pattern forming what is called epidermal proliferation unit. Stem cells divide to transit-amplifying cells that subsequently divide one to five times laterally and upward towards the epithelial surface producing a clone of differentiated cells. Eventually, the epidermal proliferation unit consists of a population of dividing progenitor (transit-amplifying) cells and their respective clones of differentiated cells [6, 9, 10]. It is also evident that proliferation and epithelial turnover increase during wound healing. Surveillance and management systems inside the cell cycle checkpoint will detect damaged cells and will subsequently activate programmed cell death [6, 9]. Exposure to environmental factors can influence the cell cycle phases of oral epithelial cells. Smoking, for example, has the ability to disrupt the cell cycle of normal epithelial cells. Michcik et al. [11] studied the influence of smoking on the cell cycle of normal epithelial cells and showed that the percentage of cells in the individual phases of the cell cycle was significantly dependant on the pack-year smoking history [11].

The oral mucosa displays normal variations that relate to its development and functional demands. Knowledge of development, biology and histology of the epithelial lining of the oral cavity sets a foundation for a better understanding of oral carcinogenesis.

Carcinogenesis

An overwhelmingly large proportion of head and neck cancers are squamous cell carcinomas (SCCs) and variants thereof, originating from the epithelium of the mucosal lining of the upper aerodigestive tract [12]. Two-thirds of these malignancies occur in developing countries; and a high incidence continues to be observed in the Indian subcontinent [13]. Many of these areas are showing rising trends, with a shift to the involvement of younger individuals, and survival rates have improved little or not at all in much of the world over several decades, mainly due to the high risk of developing a second primary cancer [14, 15]. Five-year survival rates are reportedly as low as 9% for some parts of the oral cavity, largely due to late-stage diagnosis when tumour metastasis has occurred [16]. The importance of early detection and prevention of oral cancer and pre-malignancy cannot be overstated. It is imperative to understand oral carcinogenesis and malignant transformation, for the early detection and prevention of oral cancer [4].

Carcinogenesis describes the series of genotypic and phenotypic changes that result in a cell being identified as malignant. Compared with normal cells, cancerous cells display a range of ‘hallmarks’ including resistance to antigrowth signals, evasion of apoptosis, self-sufficiency in growth signals, limitless replicative potential, promotion of angiogenesis, the ability to invade tissue and metastasise, altered

metabolic pathways and the ability to evade the immune system [17, 18]. These cancer hallmarks are functional capabilities that permit cancer cells to survive, proliferate and spread beyond their initial location. Some of these hallmark properties may become activated quite early in the process of carcinogenesis, for example, evasion of apoptosis, while others may only be present in malignant or metastatic tissues. The cancer hallmark concept also describes 'enabling characteristics' which assist in the acquisition and promotion of hallmark capabilities, namely, genomic instability and inflammation [17, 18].

Oral cancer development is a complex, multistep and multifocal process involving field cancerisation and carcinogenesis [14, 19]. In the context of oral cancer, carcinogenesis involves the cells and tissues of the normal oral mucosa transforming into oral squamous cell carcinoma. Oral cancer formation is driven by the accumulation of a series of genetic alterations which activate or inhibit various functions and signalling pathways of the normal oral mucosa, some of which are summarised in Fig. 3.1. Genetic alterations may be driven by risk factors such as tobacco smoking or alcohol consumption or by genetic susceptibility. The concept of field cancerisation, as conceived by Slaughter et al. in 1953 [20], also known as *field defect* or *field effect*, describes the process by which a large area of tissue becomes genetically but not histologically altered and is more susceptible to malignant transformation [20]. Oral cancer, like carcinomas in other tissues, develops over many years, and during this period, there may be multiple sites of neoplastic transformation

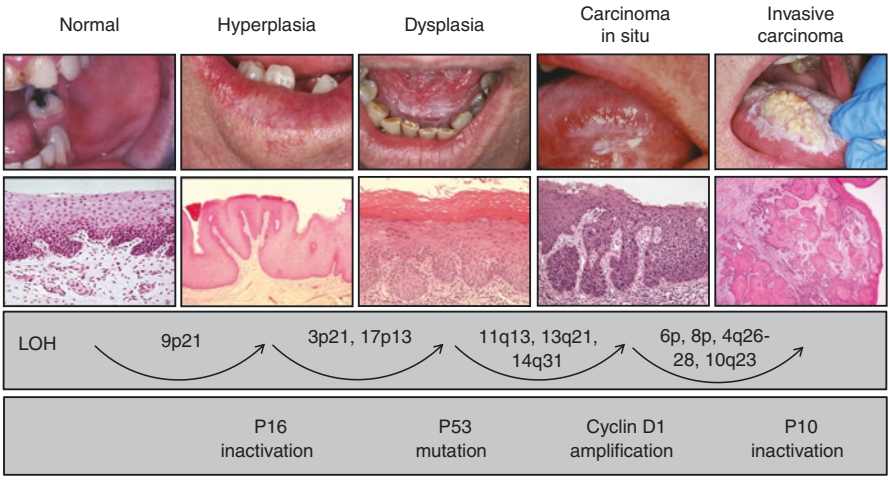

Fig. 3.1 Clinical, histological and molecular models of oral carcinogenesis. Oral carcinogenesis can be understood clinically, histologically and molecularly. The top panel depicts the physical manifestations of premalignant and cancerous lesions in comparison to normal mucosa. The images below display the histological changes that occur to normal oral mucosa as it progresses through hyperplasia, dysplasia, carcinoma in situ and invasive carcinoma. The grey boxes below depict the accumulation of mutations that occur during carcinogenesis, with characteristic regions undergoing loss of heterozygosity (LOH) (above) and critical mutations correlated with premalignant and malignant lesion stage (below). Image used with permission from C. S. Farah et al. (eds.), Contemporary Oral Medicine, Springer Nature Switzerland AG 2019

occurring throughout the oral cavity [14]. Thus, multicell anaplastic tendency results in a multifocal development process of cancer at various rates within the entire field, with subsequent development of abnormal tissues around a tumourigenic area, the areas of which may later coalesce and create atypical areas, even after complete surgical removal [21]. This may partly explain the presence of second primary tumours and recurrences [21]. The continual presence of mutations may also signify changes in DNA repair and apoptosis, thereby increasing the susceptibility to future transformation [22]. Mutational adaptations that modify the survivability of particular clones of transforming cells may also further enhance the level of resistance to therapeutic control [14, 22]. A recent genetic analysis revealed that cancers developing at distant sites within the oral cavity often are derived from the same initial clone [23]. The multiplicity of the oral carcinogenesis process makes it difficult to interrupt the progression to cancer through the surgical removal of a premalignant lesion alone [14]. It is important to recall that multifocal presentation and mutational expression of tumour suppressor genes may also be the consequence of long-term exposure to various environmental and exogenous factors [14].

Clinical Model of Carcinogenesis

Clinically, human tumours can be broadly divided into three groups: premalignant lesions, primary tumours and metastasis, the former of which is our focus. An oral premalignant lesion is an area of morphologically or genetically altered tissue that is more likely than normal tissue to develop cancer. Estimates of the global prevalence of OPMD range from 1% to 5% [24]. Cells in premalignant lesions are clonally expanded because of the acquisition of selective growth advantage by genetic alteration (or alterations) that occur in cells, and these initiated cells may be less responsive to negative growth regulators and cell differentiation inducers [21]. The initiated cells or normal cells convert to malignant cells (converted cells) by additional or multiple genetic alterations and produce primary tumours [21]. A range of tissue and cellular alterations consistent with carcinoma commonly precedes OSCC, but when it remains restricted to the surface epithelial layer, it is a potentially malignant condition termed oral epithelial dysplasia (OED) [16, 25]. Although OSCC is not linear in its development, there is general agreement that it begins as a simple epithelial hyperplasia and progresses through OED, with more severe dysplastic changes signifying more extensive genetic aberrations [16]. The timeframe for this process is not known but is thought to be a relatively slow process, with malignant transformation occurring within 10 years [16]. The reported rates of malignant transformation of leukoplakia range from <1% to 18%, and various factors, such as the location within the oral cavity, clinical appearance (homogenous versus non-homogenous) and the presence of dysplasia, correlate with the risk of progression [26].

There are clinically apparent premalignant lesions of oral cancer. They include leukoplakia, erythroplakia, oral lichen planus and oral submucous fibrosis. The

most recent definition of leukoplakia emerged from a World Health Organization-supported workshop as 'a white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer' [25]. This definition should exclude such white lesions as traumatic or smokers' keratosis, which do not carry an excessive risk of OSCC [16]. Leukoplakia is seen most frequently in middle-aged and older males, with an increasing prevalence with age. Fewer than 1% of males below the age of 30 have leukoplakia, but the prevalence increases to 8% in men over the age of 70, while the prevalence in females past the age of 70 is approximately 2% [14].

Similar to the definition for leukoplakia, erythroplakia is a clinical term that refers to a red patch that cannot be defined clinically or pathologically as any other condition. This definition should exclude inflammatory conditions that may result in a red clinical appearance. Oral erythroplakia occurs most frequently in older males and appears as a red macule or plaque with a soft, velvety texture, with the floor of mouth, lateral tongue, retromolar pad and soft palate being the most common sites of involvement [25]. Some lesions may be intermixed with white, and this is termed an erythroleukoplakia.

The most common site for intraoral carcinoma is the tongue, which accounts for around 40% of all cases in the oral cavity proper [13]. The floor of the mouth is the second most common intraoral location, with less common sites including the gingiva, buccal mucosa, labial mucosa and hard palate (Warnakulasuriya [13]). A two-tier system has been developed by Kujan et al. [27] that categorises OED into low and high risk of undergoing malignant transformation, in an attempt to make histopathology more practical for the clinician [27]. Leukoplakic lesions in high-risk sites should be considered to be at high risk of malignant transformation.

Histopathological Model of Carcinogenesis

Although diagnosis of invasive OSCC is largely uncomplicated, pathologic diagnosis of oral premalignant lesions can be perplexing. The histological finding of dysplasia is strongly associated with an increased rate of invasive cancer development. The World Health Organization (WHO) has established criteria for dysplasia, including the architectural and cytological changes in the epithelium which are summarised in Table 3.1 [28]. Visual representations of histological examples of carcinogenesis are summarised in Fig. 3.1.

The classic WHO oral dysplasia grading system includes diagnoses of no dysplasia, mild dysplasia, moderate dysplasia and severe dysplasia. In cases of mild dysplasia, cytological and architectural changes are confined to the lower third of the thickness of the epithelium; in cases of moderate dysplasia, changes are seen in up to two-thirds of the thickness of the epithelium. In cases of severe dysplasia, the dysplastic changes fill more than two-thirds of the thickness but less than the entire thickness of the epithelium. The dysplastic cells of carcinoma in situ occupy the entire thickness of the epithelium (bottom to top changes), although the basement

Table 3.1 Characteristic architectural and cytological changes for oral epithelial dysplasia grading [28]

Architectural changes	Cytological changes
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)
Abnormal mitoses not limited to basal or parabasal layers	Increased nuclear-cytoplasmic ratio
Premature keratinisation in single cells (dyskeratosis)	Increased nuclear size
Keratin pearls within rete ridges	Atypical mitotic figures
	Increased number and size of nucleoli
	hyperchromasia

membrane is still intact. Invasive SCC involves dysplastic cells invading the underlying connective tissue stroma through the basement membrane.

It should be emphasised that the assessment of dysplasia is subjective and open to interpretation, and accurate pathologic grading of dysplasia requires ample experience [29, 30]. Additionally, the merit of OED grading in predicting malignant transformation is limited by the virtue that not all OED lesions transform into malignancy [30]. On the contrary, some OED lesions may regress [24].

Animal Model of Oral Carcinogenesis

Animal models have provided a useful tool to investigate the process of carcinogenesis, the development and growth of tumours and the influence of carcinogens/risk factors and potential treatments. The use of knockout or transgenic mice is a powerful tool to understand the role of specific genes in tumour growth and development [31–33]. The most widely used animal models for oral carcinogenesis are the hamster cheek pouch model and the 4-nitroquinoline 1-oxide-(4-NQO)-induced oral (tongue) carcinogenesis model [32]. These models attempt to mimic the histological, molecular and immunological characteristics of human oral carcinogenesis as closely as possible.

Induction of SCC in the cheek pouch of hamsters was first described with the aid of three polycyclic aromatic hydrocarbons, namely, 7,12-dimethylbenz(a)anthracene (DMBA), 20-methylcholanthrene (20-MC) and 3,4-benzpyrene [14]. DMBA is a widely used carcinogen in experimental oral carcinogenesis. The DMBA model was first utilised by Salley in 1954 and involves application of DMBA (0.5%), to the hamster cheek pouch for a period of 16 weeks, which results in the formation of

invasive OSCC. The model is analogous to what is observed in humans as it shows a gradual development of precancerous lesions, which become dysplastic and undergo malignant transformation to invasive oral squamous cell carcinoma [34]. A range of variants have been developed utilising the basic model combined with other carcinogens such as alcohol, tobacco or alternate chemicals or knockout or transgenic animals.

DMBA-treated hamsters demonstrate expression of oncogenes also seen in human OSCC such as c-*erb*-B (gene for EGRF protein), c-Ha-*ras* (gene for p21 protein), Ki-*ras* and mutant p53 [35]. Expression of c-Ki-ras is of particular interest as it appears at an early stage of tumour development, but not in the healthy oral mucosa [14]. These animals display metabolic markers comparable to human lesions, namely, γ-glutamyltranspeptidase, ornithine decarboxylase and polyamines [36, 37]. A recent study performed a comparative immunohistochemical study using the hamster model and human normal and OSCC tissues and found that DMBA-induced changes in protein expression in the hamster were largely mirrored in human cancer samples. This took the form of activation of cytochrome P450, increased markers of oxidation and changes in the expression of a range of proteins involved in apoptosis, angiogenesis, proliferation and invasion [38, 39]. This model was also used to identify a novel tumour suppressor gene (TSG) named DOC-1 via a negative screen. Healthy hamster oral keratinocytes express DOC-1, while keratinocytes from DMBA-treated hamsters display mutations that lead to very low levels of DOC-1 protein [40]. Re-expression of DOC-1 in malignant oral keratinocytes results in reversion of many malignant phenotypes to normal, thus causing the DOC-1 transfected oral cancer cells to look and act like their normal counterpart [41]. The precise function of DOC-1 in normal oral keratinocyte biology is still being investigated, but it appears to act as a cell cycle regulator and be involved in apoptosis and its expression is lost in approximately 70% of human SCC [38, 42]. Other animal models for the study of oral carcinogenesis include those in rats and mice using water-soluble 4-NQO, which produce tongue lesions including squamous cell neoplasms within 32 weeks and palatal tumours within 49 weeks [14, 32]. Typically, this model includes an induction period of between 8 and 32 weeks, where 4-NQO is supplied in the drinking water, and then a development period of 15–24 weeks, where animals are fed untreated water and tongue lesions are allowed to develop. In the mouse, 10 weeks of DMBA treatment results in the majority of animals displaying lesions greater than grade 2 by week 25, with 75% of these histologically assessed as severe dysplasia, CIS or an invasive SCC [43]. In the rat, an 8-week induction generates tongue lesions including leukoplakia and papillary tumours by 32 weeks. Histologically, these lesions are predominantly hyperplastic, with some displaying mild and moderate dysplasia, but with 32 weeks of 4-NQO treatment (and 24 weeks development), the lesions display frequent severe dysplasia (80%), a 15% incidence of CIS and a rate of invasive SCC ranging from 50% to 70% [44]. Increased levels of polyamine synthesis, as well as nucleolar organiser regions (NORs), have been noted in this rat model with the progression of oral carcinogenesis [14]. Rats carrying human c-Ha-Ras proto-oncogene develop 4-NQO-induced cancer more rapidly than wild type, suggesting c-H-Ras alterations are involved in the mechanism of 4-NQO [45].

Lesions induced by 4-NQO display molecular similarities to human oral lesions. A recent RNA-seq study found increased transcript associated with matrix breakdown and migration, proliferation and cell cycle, hypoxia signalling and DNA replication compared with untreated controls [43]. This was reflected at a protein level with increased protein expression via immunohistochemistry in MMP-9, beta-catenin and markers of oxidative stress [43]. In another study by Foy et al., utilised RNA-seq to compare gene expression profiles between normal, hyperplastic, dysplastic and cancerous mouse tongue tissue generated using the 4-NQO model and correlated these with human cancer datasets. They were able to identify 'early' gene sets correlated with hyperplasia, 'intermediate' gene sets correlated with dysplasia and 'late' gene sets correlated with malignant transformation as well as a 'progressive' gene set where expression increased over the course of experimental carcinogenesis [46]. Overall, there were changes in signalling via NFKB pathway (early), the MAPK/ERK pathway (early and late), IL4 signalling and several apoptosis pathways. The 4-NQO model is characterised by frequent CASP8 mutations and amplifications at 11q13.3 and is potentially sensitive to MEK inhibition [46]. It is similar to a subset of human tumours characterised in TCGA that are HPV negative and display inactivating CASP8, activating HRAS and wild-type TP53 [47].

Molecular Model of Carcinogenesis

Genetic alterations define the molecular basis of carcinogenesis, and these include point mutations, amplifications, rearrangements, copy number variations, insertions and deletions as well as chromosomal translocations of entire exomes or genomes.

Oncogenes, gain-of-function mutations of highly regulated normal cellular counterparts (proto-oncogenes), are likely involved in the initiation and progression of oral neoplasia. Genetic damage in oral cancer cells can be divided into two categories: dominant changes most frequently occurring in proto-oncogenes but also in certain tumour suppressor genes (TSGs) result in gain of function and recessive changes, mutations most frequently noted in growth inhibitory pathway genes or commonly in TSGs, cause loss of function [21]. Cellular oncogenes were initially discovered by the ability of tumour cell deoxyribonucleic acid (DNA) to induce transformation in gene transfer assays, and these experiments have led to the identification of more than 60 cellular oncogenes [48]. Mechanism of activation of these cellular oncogenes includes point mutations and DNA rearrangements. As summarised in Fig. 3.1, several oncogenes have been implicated in oral carcinogenesis.

For human oral cancer, more than 63 karyotypes have been described and commonly reported to be associated with oral carcinogenesis among them are recurrent chromosome 9, 13, 18 and Y deletions and cytogenetic alterations in cellular oncogenes B-cell lymphoma-1, int-2 and hst which have been mapped to chromosome Iq [49]. Approximately two-thirds of all head and neck cancer cells contain a deleted region located in chromosome 9p21-22, which appears in dys-

plastic and carcinoma in situ lesions, thereby suggesting that gene in this region is knocked out early in carcinogenesis [50]. Frequently deleted also are chromosomal regions in 3p and 13q [19]. Aberrant expression of the proto-oncogene epidermal growth factor receptor (EGFR, c-erb 1), members of the ras family, as well as c-myc, int-2, hst-1, PRAD-1 and bel, is believed to contribute to oral carcinogenesis [50]. Glutathione *S*-transferase M1 (GSTM1)-null genotype appears to be the most consistent polymorphic susceptibility marker for head and neck cancer (Aida et al. [140]). *ALDH1B* and *ALDH2* (aldehyde dehydrogenase 2) genes are also associated with HNSCC and show significant correlation with alcohol consumption [51].

Califano et al. [19] demonstrated the most common allelic events in a large number of primary preinvasive lesions and invasive HNSCC to develop a molecular progression model, which involves the inactivation of many putative suppressor gene loci. Chromosomes 9p and 3p appear to be lost early, closely followed by loss of 17p [52]. Mutations in p53 gene are seen in the progression of preinvasive to invasive lesions. Other genetic events, such as amplification of cyclin D1 and inactivation of p16, have been tested predominantly in invasive lesions, but their precise order in the model was not determined.

More than 50% of all primary HNSCC harbour a p53 mutation, and inactivation of p53 represents the most common genetic change in all human cancers. The importance of p53 in a larger percentage of cancers may be shown following identification of members of its suppressor pathway, which themselves may be altered. In normal cell biology, p53 acts as a regulator of DNA synthesis, and when genomic DNA is damaged, p53 is produced to block cell division at the G1-S boundary and stimulate DNA repair and activate pathways leading to apoptosis [19]. Mutation of p53 allows a tumour to pass through G1-S boundary and propagate genetic alterations that lead to other activated oncogenes or inactivated TSGs [19]. The most commonly deleted region in head and neck cancer is located at chromosome 9p21–22 [53]. Loss of chromosome 9p21 occurs in the majority of invasive tumours in head and neck cancer, and frequent homozygous deletions in this region represent one of the most common genetic changes identified [53]. p16 (*CDKN2*) present in this deleted region is a potent inhibitor of cyclin D1, and this loss of p16 protein has also been found in most advanced premalignant lesions and is important in early malignant progression [54]. The loss of chromosome 17p is also frequent in most human cancer and is seen in approximately 60% of invasive lesions [52]. Although p53 inactivation correlates closely with loss of 17p in invasive lesions, p53 mutations are quite rare in early lesions that contain 17p loss [52]. Loss of chromosome arm 10 and 13q is also noted in primary tumours [19].

Loss of function of the tumour suppressor p53 can result in uncontrolled cell division and progressive genomic instability, such as the loss of heterozygosity (LOH) and microsatellite instability (MSI).

Chromosome 9p21 containing p16 tumour suppressor gene is frequently lost in HNSCC and oral preneoplastic lesions. Chromosome 3p14 contains the tumour

suppressor gene fragile histidine triad (FHIT) as well as a common fragile site, FRA3B which is also found to be frequently deleted in early tumourigenesis, and its deletion is associated with exposure to cigarette smoke [55].

Stimulating oral keratinocyte proliferation are growth factors, and during oral carcinogenesis, growth factors are deregulated through increased production and autocrine stimulation [49]. Transforming growth factor-alpha (TGF-alpha) is overexpressed early in oral carcinogenesis by hyperplastic epithelium and later by the inflammatory infiltrate, particularly eosinophils, surrounding the oral epithelium, and in head and neck cancer patients who later develop a second primary cancer, normal oral mucosa oversecretes TGF-alpha, suggesting a premalignant state of rapid proliferation and genetic instability of the epithelium [49]. Concomitant expression of TGF-alpha and EGFR may indicate more aggressive tumours than those overexpressing EGFR alone [49].

EGFR is the biological receptor of EGF and TGF-alpha, and malignant oral keratinocytes possess between 5 and 50 times more EGFR than their normal counterparts [56]. Oral tumours overexpressing EGFR exhibit a higher proportion of complete responses to chemotherapy than tumours with low-level EGFR expression. Overexpression of EGFR, presumably due to higher intrinsic proliferative activity, could result in higher sensitivity to drug therapy cytotoxic to cells undergoing mitogenesis [56]. Though not fully understood, several mechanisms have been postulated to activate EGF genes in carcinogenesis [49] including deletions or mutations in the N-terminal ligand-binding domain such as those occurring in the viral oncogene verb B, overexpression of the EGFR gene concurrent with the continuous presence of EGF and/or TGF-alpha or deletion in the C-terminus of the receptor, which prevents downregulation of the receptor after ligand binding.

Also important in inhibiting oral keratinocyte proliferation are cell surface molecules such as E-cadherin, a cell-to-cell adhesion molecule associated with both division and metastasis, which is downregulated in oral cancers, and DOC-1, an N-Cam-like molecule believed to be an important cell-to-cell contact inhibitor that is mutated during oral cancer development [40].

Transcriptional factors, or proteins that regulate the expression of other genes, are also altered in oral carcinogenesis, and this alteration of intracellular pathways modulates gene expression. The transcription factor c-myc, which helps to regulate cell proliferation and differentiation, is frequently overexpressed in oral cancer, and this is most frequently associated with poorly differentiated tumours and with poor prognosis (Shpitzer et al. [50]). Genes whose expression is stimulated by c-myc and their significance to oral carcinogenesis are being studied [49]. Also amplified in oral cancer is another important transcription factor, the cell cycle promoter PRADI (also CCNDI or cyclin Dl), whose importance to oral carcinogenesis is being investigated [48].

Growth suppressor intracellular messengers may include the adenomatous polyposis coli (*APC*) gene, a G-like protein frequently mutated in certain familial colorectal cancers, and the *APC* gene may be altered in premalignant oral lesions [50].

Factors Influencing Oral Carcinogenesis

Risk Factors

The incidence of oral cancer has significant local variation. The geographical patterns of oral cancers have been presumed to be due to the varying prevalence of risk factors among countries, in particular tobacco and alcohol consumption and the diet quality [57]. Similarly, the variations by ethnicity are largely due to the social and cultural practices and the influence of dietary and genetic factors [57]. Numerous risk factors or possible causative agents have been described, which are covered in detail in other chapters. It is important to note that the presence of one risk factor enhances the effects of a second risk factor and development of OPMD and oral cavity cancer. This concept is termed synergism. In a study by Kadashetti et al. [58], an odds of 2.2 times more of developing OPMD cases was observed for a combination of risk factors, that is, smoking, tobacco quid chewing and alcohol drinking, as compared to non-chewers, non-smokers and non-drinkers [58].

While it is traditionally assumed that OPMD risk factors are similar to OSCC risk factors, a proportion of OPMD and OSCC cases occur in the complete absence of any identifiable risk factor, particularly in young never smokers affected by these diseases [59]. Our understanding of OPMD aetiology is incomplete, and there is an urgent need for further research into predisposing factors.

A cross-sectional study by Kumar et al. [60] in 2015 found that 13.7% of the Indian population studied showed the presence of OPMD, and of these, oral submucous fibrosis was the most prevalent and erythroplakia the least prevalent. In this study, males were found to have a significantly higher prevalence of OPMD compared to females, presumably due to an increased number of males with smoking and smokeless tobacco usage. No significant differences were found on socioeconomic status, toothbrushing methods or brushing frequency [60].

Tobacco and Smokeless Tobacco

The various forms of tobacco use can vary across geographical areas and cultures around the world, and in the United States, Europe and Australia, cigarettes, cigars and pipes are the major types of smoking tobacco, while chewing tobacco and areca nut, also known as betel nut, are the most common forms of smokeless tobacco, predominantly used in India, Pakistan, China and other areas of Asia. Smokeless tobacco is used either alone or as part of a concoction and in a myriad of forms including betel quid, bidis, paan, naswar or nass [61]. Although the use of electronic nicotine delivery systems (or ‘ENDS’), also known as e-shisha, e-cigars, e-pipes, e-Hookas, hookah-pens, vape-pipes and e-cigs are commonly advocated as a means of tobacco harm reduction [62], although there is building evidence that they are not safe and may still contribute to oral carcinogenesis causing DNA strand breaks and cell death [63, 64].

Smoking and smokeless tobacco use are well-accepted risk factors for developing oral cancer and are implicated in a large majority of squamous cell carcinomas in the head and neck region. The risk for squamous cell cancer of the head and neck is estimated to be approximately tenfold greater for current smokers compared with never smokers [65]. This risk decreases with time from cessation of exposure, although the risk never reduces to the level of a never smoker [65]. For persons with an OPMD relative to individuals with a benign oral tissue condition, Li reported that the adjusted OR for current smoking was 4.32 (95% CI, 1.99–9.38), while for former smokers, the OR was 1.47 (95% CI, 0.67–3.21), each OR relative to never smokers [66]. A reduction in tumour suppressor activity by the gene and the development of mutations in p53 are associated with smoking and an increased risk for oral carcinoma development [14, 49]. Across anatomic tumour sites, the NFE2L2 oxidative stress pathway is a tobacco-related signature. Areca nut, also known as betel nut, use has been associated with the development of OPMD, and various studies from around the world have reported the adverse effects of areca nut chewing. Some areca nut-specific nitrosamines suspected to be carcinogenic are 3-methylnitrosamino propionaldehyde (MNPA), 3-methylnitrosamino propionitrile (MNPN), *N*-nitrosoguvacine (NGC) and *N*-nitrosoguvacoline (NGL). MNPA in particular causes DNA single-strand breaks and DNA protein cross links [60].

The literature reports an odds ratio of between 8.4 and 41 for developing OPMD in tobacco/areca nut chewers as compared to non-chewers [58]. The highest odds ratio is found in India, and it is plausible that this difference could be due to the differences in habits practiced by different study populations and also the composition and method of chewing which varies from country to country [58]. As the duration of tobacco/areca nut chewing increases, the risk of developing OPMD and oral cancer increases in a dose-dependent manner [58]. A recent systematic review of 18 case-control studies reported that betel quid with tobacco chewing carried the highest risk for developing OPMD compared with other forms of smokeless tobacco use [67].

Alcohol

Numerous studies have suggested that alcohol is a risk factor for oral cancer. Individuals consuming more than 170 g of whisky daily have ten times higher risk of oral cancer than light drinkers [68]. Alcohol may have an additive effect, and it has been suggested that it facilitates the entry of carcinogens into exposed cells, altering the metabolism of oral mucosal cells [68]. Therefore, alcohol consumption and tobacco use can have a synergistic effect on cancer risk. It has been suggested that the incidence of p53 mutation is much higher in patients who are exposed to both tobacco and alcohol versus non-users. A study in 2008 implicated the ADH1C*2/*2/MTHFR 677TT genotype combination as more susceptible for developing OSCC, with a 20-fold increase in risk in heavy drinkers and a 5.9- and 2.8-fold increase in risk, respectively, in moderate drinkers and light drinkers [69].

Comparatively, few epidemiologic studies, however, have investigated the role of alcohol in relation to OPMD risk [66]. Findings from investigations into the association between alcohol consumption and either oral leukoplakia or other potentially premalignant oral diagnoses have not been consistent across the studies [70]. There is uncertain consensus that overall alcohol consumption is associated with increased OPMD risk. Some studies of premalignant lesions and OED have found that risks associated with drinking are dependent upon the level of alcohol intake, with the highest risks observed for the highest level of alcohol intake; however, after adjusting for various potential confounders, one study found no evidence of an increased OMPD risk even among those persons who consumed >20 drinks/week [66]. Previous studies have reported that the risk of oral cancer and OPMD can vary by beverage type (beer, wine and hard liquor); however, that same study found little evidence that any type of alcoholic beverage consumption was associated with an increased OPMD risk [66, 70]. In contrast, a risk factor model reported that betel-quid chewing and consumption of alcohol were the only statistically significant characteristics for OPMD risk after controlling for other factors [71]. After controlling for all other variables, the adjusted OR for daily chewers was 10.1 (95% CI, 3.4–29.7), with a strong dose-response relation, and when considering the consumption of alcohol and risk of OPMD, the adjusted OR for weekly drinkers was 2.7 (95% CI, 1.2–6.3) [71].

The risk of oral cancer formation in chronic users of alcohol containing mouth rinses is controversial [72], but there appears to be building evidence that the use of high alcohol-containing mouthwashes has a synergistic effect with tobacco smoking and is more likely to add to the risk profile of patients displaying OED [73], with a greater proportion of mouth rinse users displaying dysplasia on histopathology, although this did not reach statistical significance [74].

Viruses

Much research is being performed to determine the role of oncogenic viruses in human cancer, and it is an emerging area of study. Viruses are capable of hijacking host cellular apparatus and modifying DNA and chromosomal structures and inducing proliferative changes in cells [68]. In particular, the human papilloma virus (HPV) and the herpes simplex virus (HSV) have been implicated in forms of oral cancer.

The role of some HPV subtypes in the aetiopathogenesis of OPMD is controversial; however, an epidemiological association between HPV and OMPD has been reported [75]. A systematic review found that HPV detection rate is higher in the OPMD group than in the controls (OR = 3.87; 95% CI, 2.87–5.21), suggesting a potentially important causal association between HPV and OPMD [75]. The most commonly detected HPV in the head and neck squamous cell carcinoma (HNSCC), detected in 90–95% of HPV-positive cases, is HPV-16, followed distantly by HPV-18, HPV-31 and HPV-33 [68]. The HPV type most commonly detected in OPMDs has been reported to be HPV-16, 18 with HPV-6, 11 found in only a few studies

[75]. In a subgroup analysis of OPMD, Syarjanen et al. calculated the pooled estimates of the odds ratio (OR) for the association of HPV with OPMD, when compared with healthy oral mucosa, and reported that HPV was associated with oral leukoplakia (OR = 4.03; 95% CI, 2.34–6.92), oral lichen planus (OR = 5.12; 95% CI, 2.40–10.93) and epithelial dysplasia (OR = 5.10; 95% CI, 2.03–12.80) [75]. HPV-positive oral and oropharyngeal cancer are a distinct clinico-pathological entity and are less likely to occur among heavy smokers and drinkers and have lesser likelihood of p53 mutation. While the prognostic significance of HPV in precancerous oral lesions is not clear, most studies have reported improved disease-specific survival and a better prognosis for HPV-positive oropharyngeal cancer [68]. It has been suggested that HPV-positive tumours may have better prognosis by inactivating retinoblastoma (Rb) [68].

Similarly, the role of HSV in the aetiopathogenesis of OPMD is controversial; however, there may be an epidemiological association between HSV and OMPD [76]. Epidemiological studies have showed higher levels of IgG and IgM antibodies in oral cancer patients as compared to control subjects. A population-based study reported that HSV1 antibody positivity was associated with a slightly increased risk of OSCC (adjusted odds ratio (OR), 1.3; 95% confidence interval (CI), 0.9–2.0) and concluded that the presence of HSV1 may increase the risk of OSCC in individuals who were already at increased risk due to cigarette smoking or HPV infection [77].

Risk of oral cavity and pharyngeal cancer is twofold higher among human immunodeficiency virus (HIV) patients indicating an association between HIV and OSCC [68]. Epstein-Barr virus (EBV), human herpesvirus-8 (HHV-8) and cytomegalovirus have also been reported as risk factors of OSCC in different studies [68]. A recent study assessing the immunohistochemical expression of Epstein-Barr virus latent membrane protein 1 among OPMD, OSCC and healthy controls found that there was no significant association between Epstein-Barr virus positivity and OPMD and OSCCs [78].

Candida

Candida species have been suggested to play a role in oral carcinogenesis; in particular, *C. albicans* have been implicated in the development of OPMDs. However, the pathogenesis is not well understood and is still a field under research.

The ability of *C. albicans* to colonise, penetrate and damage host tissues depends upon the imbalance between *C. albicans* virulence factors and host defences, often due to specific defects in the immune system [79]. Cell surface proteins called adhesins mediate adherence of *C. albicans* to other microorganisms and host cells. The contact to host cells triggers phenotypic switching from the yeast form to hyphae form, which directs production of carcinogenic compounds, like the nitrosamine *N*-nitrosobenzylmethylamine [80]. Strains with high nitrosation potential have been isolated from lesions with more advanced precancerous changes, and the yeast cells in such cases extend from the mucosal surface to the deeper epithelial cell layers, representing transport and deposition of precursors (like nitrosamines)

to deeper layers [80]. Carcinogenic compounds can then bind with DNA to form adducts causing miscoding or irregularities in DNA replication. Certain strains of *C. albicans* may play a key role in the development of dysplasia [80].

It has also been shown that the epithelium of the chick embryo, when infected with *C. albicans*, shows squamous metaplasia and a higher proliferative phenotype [68]. Leukoplakia with candidal infection has been reported to have a higher rate of malignant transformation than uninfected leukoplakia [81]. The causal association of *Candida* species and oral cancer is controversial and requires further study.

Inflammation

Cytokines seen in inflammation, including interleukins (ILs), tumour necrosis factors (TNFs) and certain growth factors, are an important group of proteins that regulate and mediate inflammation and angiogenesis, and when there is a downregulation in their production, tumour growth, invasion and metastasis are facilitated [14]. A putative correlation has been raised by some genetic association studies between functional DNA polymorphisms in cytokine genes and oral cancer [14]. When compared to controls, patients with oral cancer demonstrate increased serum levels of proinflammatory cytokines, interleukin (IL)-1β, IL-6, IL-8 and TNF-α, as well as the anti-inflammatory cytokine, IL-10 [14]. The anti-inflammatory cytokine IL-4 inhibits oral cancer invasion by the downregulation of matrix metalloproteinase-9 [14].

Genetic Predisposition

Genetic predisposition and family history have been shown to play a role in head and neck cancer, related to polymorphisms in carcinogen-metabolising enzyme systems. A recent extensive meta-analysis pooled individual-level data across 12 case-control studies including 8967 HNC cases and 13,627 controls; after adjusting for potential confounding factors, a family history of head and neck cancer in first-degree relatives increased the risk (OR = 1.7, 95% CI 1.2–2.3) [57]. The risk was higher when the affected relative was a sibling (OR = 2.2, 95% CI 1.6–3.1) rather than a parent (OR = 1.5, 95% CI 1.1–1.8) and for more distal head and neck sites (hypopharynx and larynx) [57]. The OR rose to 7.2 (95% CI 5.5–9.5) among subjects with a family history, who were alcohol and tobacco users, and no association was observed for a family history of nontobacco-related neoplasms and the risk of HNC (OR = 1.0, 95% CI 0.9–1.1) [57]. Other estimates of risk in first-degree relatives of head and neck cancer patients range from 1.1 to 3.8 [59].

Polymorphic variation of genes in the xenobiotic metabolism pathways such as in *CYP1A1* or the genes coding for glutathione *S*-transferase-M1 and N-acetyltransferase-2 may be implicated [59]. Individuals that carry the fast-metabolising alcohol dehydrogenase type 3 (*ADH3*) allele may be particularly vulnerable to the effects of chronic alcohol consumption and could be at increased risk

to develop oral cancer [59]. The single-nucleotide polymorphism A/G870 in the *CCND1* gene that encodes Cyclin D is associated with susceptibility to oral cancer. The AA genotype or the GG wild-type genotype may increase risk for oral cancer [59].

A recent review reported increased susceptibility for OPMD risk with single-nucleotide polymorphisms (SNPs) in *GSTM1 (null)*, *CCND1 (G870A)*, *XPD (codon 751)* and *MMP3 (-1171; promotor region)*, common in majority of populations (Asians, Caucasians, Brazilians and others) [82]. However, the risk associated with SNP in *p53 (codon 72)* was restricted to Indian populations, and it was hypothesised that the high prevalence of SNP in *p53 (codon 72)* may be partly responsible for higher incidence of OPMD in this population [82]. It is possible this may be a chance association, with p53 being the most commonly inactivated tumour suppressor gene in the development of oral cancer. *Gemin3 (rs197412 C/T)* on the other hand was found to be associated with reduced risk for OPMD in Indian and Caucasian populations [82].

Genetic Instability Syndromes

A cancer syndrome is a genetic disorder in which inherited generic mutations predispose affected individuals to developing multiple independent primary tumours, and these individuals carry a high lifetime risk of developing cancer. Tumour suppressor genes are involved in controlling cell growth, both by acting as gatekeepers and inhibiting cell proliferation and promoting cell death, and by acting as caretakers, maintaining the integrity of the genome by DNA repair mechanisms [83]. Mutations of these genes are implicated in the development of cancer syndromes, and other genes that may be affected include DNA repair genes, oncogenes and genes involved in angiogenesis.

There are several genetic diseases that have a genetic instability phenotype and a higher frequency of carcinogenesis. In disorders such as xeroderma pigmentosum, ataxia-telangiectasia, Bloom's syndrome and Fanconi anaemia, where there are defective caretaker genes, there is an increased incidence of second primary malignancies, including oral cancer (Prime et al. [83]). By contrast, with the exception of Li-Fraumeni syndrome, abnormalities of gatekeeper genes do not usually predispose to oral cancer [83]. The protein produced by the *TP53* gene, p53, is involved in cell cycle arrest, DNA repair and apoptosis [84]. Defective p53 may not be able to properly perform these processes, which may be the reason for tumour formation in Li-Fraumeni patients. Because only 60–80% of individuals with the disorder have detectable recessive mutations in *TP53*, other mutations in the p53 pathway may be involved. These include MDM2 overexpression and *CDKN2A* deletion [83].

About 1% of the general population is heterozygous for *ATM* mutations such as that noted in Ataxia telangiectasia (AT) patients [84]. *ATM* is important in activating p53 in response to DNA damage. Variants of AT are caused by mutations in *NBS* and in MRE11A [84].

Bloom's and Werner's syndromes have a defect in genomic stability in common. The genes mutated in these syndromes, *BLM* and *WRN*, respectively, are highly homologous to RecQ helicase. The predominant form of mutations is gross DNA deletions [84]. Both *BLM* and *WRN* are associated with processing the structures associated with stalled replication forks [84].

The cells from patients with Fanconi anaemia (FA) display high levels of chromosomal instability and are hypersensitive to mitosis-inducing cross-linking agents [84]. The genes known to cause FA are FANCA, FANCB, FANCC, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ, FANCL, FANCM, FANCN, FANCO, FANCP and BRCA2 (previously known as FANCD1) [84]. Damaging mutation in any of these genes inhibits the efficacy of the core FA protein complex and limits its ability to act in DNA repair. The FA pathway is involved in DNA repair when the two strands of DNA are incorrectly joined together, a process also known as interstrand cross linking [84]. The FA pathway also affects many other pathways, such as nucleotide excision repair and homologous recombination [84].

Genetic mutations in four DNA mismatch repair genes (*MLH1*, *MSH2*, *MSH6* and *PMS2*) and the EPCAM gene have been implicated in Lynch syndrome [85]. Defective *MMR* genes allow continuous insertion and deletion mutations in regions of DNA known as microsatellites, leading to a state of microsatellite instability [85]. Mutated microsatellites are involved in tumour initiation and progression and prevent the proper repair of DNA prior to cell division, allowing abnormal cells to divide and thus increasing the risk for cancer [85].

Xeroderma pigmentosum (XP) may be caused by genetic mutations in eight genes, which produce the following enzymes: XPA, XPB, XPC, XPD, XPE, XPF, XPG and Pol η [84]. XPA and XPF are nucleotide excision repair enzymes that repair UV light-damaged DNA, and faulty proteins will allow the build-up of mutations caused by UV light [84]. Pol η is a polymerase which replicates UV light-damaged DNA, and mutations in this gene will produce a faulty pol η enzyme that cannot replicate DNA with UV light damage [84].

Immune System and Host Response

The immune system plays a key role in the progression of head and neck cancer, and a greater understanding of its contribution will lead to better therapies and improved patient outcome.

Immune surveillance is the destruction of nascent cancer cells by the immune system before tumour formation can occur [57]. Immune system derangements or alterations in transformed cells may allow immune escape that allows the cancer to become manifest [57]. There are global alterations in the functional state of the immune system, as evidenced by changes in serum cytokines, chemokines and other immune-related biomarkers in cancer patients. Cancer cells evade the immune system by two primary mechanisms: by reducing their innate immunogenicity or by suppressing the immune response. Tumour cells can reduce T-cell-mediated recognition by altering HLA class I expression, and it has been noted that some tumour cells have a complete

loss of HLA expression due to defects in b2-microglobulin expression or function [57]. Alternatively, chromosomal defects in HLA-encoding genes themselves can cause selective loss of HLA expression, and this process has been noted in approximately 50% of head and neck squamous cell carcinomas and is correlated with poor prognosis in oesophageal and laryngeal squamous cell cancers [57].

In head and neck cancer, circulating serum antibodies have been found against p53, MUC1, p40, p73 and HPV E6 and E7 [57]. High postoperative levels of anti-p53 antibody have been correlated with poor prognosis. Of unclear significance is whether the levels of circulating antibody have any correlation with clinical outcome and whether the reported increase in frequency of IgE subtype immunoglobulins in head and neck cancer is of any importance. In developed head and neck cancers, an endogenous host immune response is prognostic, as has been demonstrated for multiple tumour types: T-cell infiltration of both CD4+ and CD8+ populations have been found to be prognostic in tonsillar and base of tongue SCCs, lymph node-infiltrating CD8+ T cells as well as CD20+ B cells were found to be prognostic in both oropharyngeal and hypopharyngeal cancers, and peritumoural CD8+ T cells in oral cancer have been found to be associated with lymph node metastases, tumour size and clinical stage [86].

In other cancers, there is ample expression of HLA and tumour antigen but without recognition by T cells [57]. Because HLA loss variants are killed by NK cells, one proposed explanation for the lack of NK cell killing is that cancer cells possess defects in their antigen presentation machinery (APM) as this would reduce selectively tumour antigen-HLA peptide completely without reduction in overall surface HLA density [57]. Antigenic peptides are transported to the endoplasmic reticulum by the transporter associated with antigen processing (TAP) where they are associated with HLA class I heavy chains by tapasin [57]. Thus, HNSCC cells that express HLA I and whole tumour antigen can evade T-cell recognition through decreased expression of LMP2, TAP1, TAP2 and tapasin [57]. In addition to decreased expression of HLA, HNSCC tumour cells express Fas ligand which can interact with Fas and transduce a powerful apoptotic signal to activated T cells allowing immune evasion by eliminating tumour-infiltrating T lymphocytes [57]. As mentioned, decreased expression of HLA molecules is protective against T cells but increases NK cell-mediated cytolysis, as the absence of HLA removes a key inhibitory signal for NK cells, and tumour cells must therefore employ multiple mechanisms to suppress NK cell-mediated antitumour immunity. MICA, a ligand of NKG2D in NK and T cells, can be released in a soluble form to act as a competitive antagonist [57]. Known to be produced by tumour cells are immune-suppressive cytokines and other molecules such as IL-10, TGF-b, IL-6, PGE, VEGF and GM-CSF [57]. IL-10 reduces activation of cytotoxic T cells and has been correlated with advanced-stage head and neck cancer; TGF-b suppresses T-cell and NK activation and is a key cytokine in the differentiation of regulator T cells; TGF-b production is increased in preneoplastic oral cavity lesions and promotes angiogenesis and a protumourigenic microenvironment linking it to early tumour formation. Transcription factors such as NFk(kappa)B (nuclear factor kappa-light-chain-enhancer of activated B cells) and STAT3 (signal transducers and activators of transcription), which are usually dysregulated in tumour-promoting inflammatory states in response to cytokine

stimuli, are aberrantly activated in tumour cells and are intensively studied as possible targets for therapeutic intervention [57]. Tumours themselves produce cytokines such as TGF-b(beta), IL-6 and IL-10, which suppress cell-mediated antitumour immunity [57]. In response to inflammatory stimuli, head and neck cancer cells also express receptors which are involved in lymphocyte and dendritic cell migration [57]. Expression of these receptors by tumour cells, such as CCR7 and CXCR4, constitutes immune exploitation of established signals intended for immune cells and has been associated with tumour invasion, metastasis and cell survival, leading to treatment resistance [57].

The role of the immune system in the development of all head and neck malignancies is evident in virally mediated cancers such as HPV-associated oropharyngeal tumours and EBV-associated nasopharyngeal cancers [86]. While both EBV and oral HPV infections are on the rise, nasopharyngeal and oropharyngeal cancers develop less frequently, and this is presumably due to the failure of the immune system to remove these oncogenic infections [86]. The immune checkpoint ligand programmed cell death (PD)-L1 has been identified in tonsillar crypts irrespective of HPV infection, and PD-1+-infiltrating lymphocytes are found in both chronic tonsillitis and HPV-associated oropharyngeal tumours; once HPV infection is established, multiple immune-inhibitory mechanisms, including activation of the PD-1/PD-L1 axis, may contribute to T-cell dysfunction and exhaustion [86]. Head and neck cancers not associated with HPV infection likely also co-opt immune regulatory mechanisms to facilitate their progression. Increased PD-L1 expression similarly has been detected in tobacco- and alcohol-induced SCC of the head and neck as well as other virally mediated tumours, including nasopharyngeal carcinoma and natural killer T (NKT)-cell lymphoma [86].

Premalignant lesions may be immunogenic and targetable with immunologic therapies to prevent progression to malignancy. For example, increased PD-L1 expression has been demonstrated on actinic cheilitis as well as respiratory papilloma lesions which can progress to larynx cancer. Although not necessarily indicative of a premalignant condition, a systemic antibody response directed against the oncogenic HPV E6 and E7 proteins has been demonstrated to be highly specific for the eventual diagnosis of oropharyngeal cancer, and this includes antibody responses that predate oropharyngeal cancer diagnosis by several years [86]. These antibody titers could be used to identify those at highest risk for inclusion in surveillance protocols, and in a similar manner, IgA antibody responses directed against EBV antigens have also been investigated for their ability to aid in the diagnosis of nasopharyngeal cancers [86].

Malignant Transformation

Genetic Alterations in Oral Cancer

The typical solid cancer harbours between 33 and 66 somatic mutations likely to produce changes in protein expression, and OSCC is at the high end of this spectrum with 66 mutations, although not as dramatic as melanoma or lung cancer which involve potent mutagens (approx. 150) or MSI colorectal tumours displaying

deficiencies in DNA damage repair genes [87]. It has been estimated that each driver mutation will increase the likelihood of cell survival by just 0.4% but that this growth advantage will become important with increasing mutational burden and time [87].

Large-scale sequencing efforts such as the Cancer Genome Atlas (TCGA) have allowed a greater understanding of the cancer genome and permitted classification of OSCCs that may be histologically similar on the basis of their genetic differences [47]. Table 3.2 (adapted from Kang et al. [88]) summarises the gene mutations

Table 3.2 Gene mutations in head and neck squamous cell carcinoma identified by The Cancer Genome Atlas

Mutated gene	Protein	Cancer gene class	% Tumours with mutation in TCGA (n = 279)	Hallmark involvement
*TP53**	p53	TSG	72	Evasion of growth suppressors and apoptosis, proliferative signalling
*NOTCH1**	Notch1	TSG	19	Evasion of growth suppressors and apoptosis, proliferative signalling
*CDKN2A**	p16 and p14arf	TSG	22	Proliferative signalling, apoptosis (p14arf)
*PIK3CA**	p110a	Onco-gene	21	Proliferative signalling
FBXW7	F-box/WD repeat-containing protein 7	TSG	5	Targets cyclin E for degradation, controls Notch1 stability
*HRAS**	p21, H-Ras	Onco-gene	4	Growth factor signalling, proliferation
SYNE1	Syne-1	NA	18	Cytoskeleton, centrosome migration
*FAT1**	Proto-cadherin Fat1	NA	23	Cadherin, Wnt signalling
KMT2D (MLL2)	KMT2D	TSG	18	Histone methylation (immortalisation)
*CASP8**	Caspase 8	TSG	9	Apoptosis
PTEN	PTEN	TSG	2	Protects against genomic instability, controls proliferation, apoptosis
NSD1		NA	10	Transcription factor
KMT2C (MLL3)	Lysine methyltransferase	TSG	8	Histone methylation (immortalisation)
EP300	P300	TSG	7	Regulates proliferation differentiation

The table summarises 14 of the most prevalent gene mutations identified in a large-scale screen of 279 head and neck cancers performed by The Cancer Genome Atlas consortium [47, 114]. As well as the mutated gene and its prevalence in the TCGA cohort, the table includes the protein coded for by the gene and where the gene/protein fits in terms of cancer hallmarks and classifies genes as either tumour suppressor genes (TSG), oncogenes or neither (NA). Genes highlighted with an asterisk have been demonstrated to occur specifically in OSCC, although generally present at a lower prevalence than in the entire HNSCC TCGA cohort [163, 164]

found by TCGA [47] and outlines the functions and cancer hallmark associations of the proteins they code for.

TCGA has provided an unprecedented level of characterisation of the mutational landscape of advanced cancers, which has driven novel treatments and improved patient care. However, it is recognised that the most effective interventions are those that target cancer at an early stage, prior to malignant transformation. In order to do this effectively, there has been a recent call for a concerted research effort towards the development of a 'Pre-Cancer Genome Atlas' (PGCA) [89, 90]. The PCGA calls for the 'comprehensive genetic profiling of premalignant lesions' performed over time, with associated histological results and outcomes [89]. This information, when applied to oral premalignant lesions, would allow the production of a catalogue of driver genes for OSCC. This in turn would allow more accurate stratification of low- and high-risk OPMLs, and personalised treatment as new immuno- and chemotherapeutics becomes available. This approach underpins our current research efforts, but significant advances in this domain will require international collaboration and scientific will to tackle the oral premalignant disease problem, as this represents an ideal model for understanding pre-malignancy while concurrently driving the PCGA agenda. The recent 'Erlotinib Prevention of Oral Cancer' (EPOC) trial provided the first example of 'high-risk' OPML being treated chemotherapeutically in an attempt to prevent progression to OSCC, and while no benefit was shown for the treatment drug, it did provide a valuable proof of concept approach [91].

The molecular characterisation of oral carcinogenesis has not been fully defined; however, it has been well-documented in the case of colon cancer. In this cancer, the first 'gatekeeper' mutation is typically in the tumour suppressor gene *APC* (adenomatous polyposis coli), which provides a selective advantage to the cell allowing it to outcompete its surroundings and start to produce a small, slow-growing adenoma [92]. A second mutation is characteristically in the gene *KRAS*, which produces an important signalling molecule and allows cells carrying both mutations to proliferate. Further mutations in genes such as *PIK3CA*, *SMAD4* and *TP53* give the cells the capacity to invade basement membrane and metastasise [87].

Biomarkers of Malignant Transformation

Oral cancer is frequently preceded by oral potentially malignant lesions (OPMLs); however, a comparatively small proportion of OSCCs, between 5% and 15%, will undergo malignant transformation [16]. Histopathology is currently the gold standard to identify and monitor OPMLs for the risk of malignant progression; however, given the invasiveness and variability of this technique, there is great interest in the identification of biomarkers that are able to segregate progressive from non-progressive OPML.

A biomarker can be defined as 'a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or

pharmacologic responses to a therapeutic intervention' [93]. Biomarkers can be prognostic (information of prognosis and the potential of recurrence), diagnostic (detection) or predictive (predict response to treatment). We will focus on biomarkers that are useful in both identifying OSCC and predicting which OPMLs are likely to convert to OSCC. We will include markers of genomic instability, gene and protein biomarkers and epigenetic modulators.

Measures of Genomic Instability (CNV and LOH)

Genomic instability underlies carcinogenesis and malignant transformation, and assessment of genomic integrity has been utilised as a biomarker of malignant transformation. Changes in DNA content/ploidy, copy number variation and loss of heterozygosity are all manifestations of genomic instability that occur over the process of carcinogenesis, and particular patterns of these can be used to identify malignant transformation [18].

DNA content/ploidy can be used as a surrogate marker of genomic damage and is measured by flow or image cytology. Tobacco smoking induces aneuploidy in normal oral mucosa [94], and leukoplakia has been correlated with changes in ploidy [95].

Copy number variation describes abnormal duplication or insertions across the genome and is a measure of genomic instability. Characteristic copy number variations of −8p/+3q/+8q have been identified in oral cancer but are also present in oral epithelial dysplasia suggesting they may be an early step in malignant transformation. HNSCC in smokers displays a high prevalence of copy number changes particularly amplifications of 3q26/28 and 11q13/22 [47]. A recent study investigated copy number changes in tumour margins and was able to correlate changes in chromosomes 1 and 7 with tumour recurrence. Copy number changes in at least one tumour margin resulted in a higher risk of local recurrence and a decreased 5-year recurrence-free survival rate (47.1% vs. 88.9%) [96].

Loss of heterozygosity (LOH) describes a loss of genomic material, in a somatic cell, at a heterozygous region so that only one copy of a gene remains. In the case of cancer, where mutations are frequent, this can leave the remaining copy of a tumour suppressor gene, for example, vulnerable to inactivating mutation.

OSCC is characterised by chromosomal gains at 1q, 3q, 5p, 7p, 8q, 9q, 11q, 14q and 18p and losses at 3p, 4p, 4q, 5q, 8p, 9p, 10p, 11q, 13q, 17p, 18q and 21q [97, 98]. Malignant transformation has been correlated with LOH 3p and 9p (as well as 8p, 11q, 13q and 17p), and the transition to invasive carcinoma has been associated with LOH (6p, 8p, 4q 26–28, 10q23). Leukoplakia with LOH at 3p and 9q display a 3.8-fold increase in malignant transformation, and this is present in approximately 50% of leukoplakia [99]. A further study found LOH at 9p21 and/or 3p14 in 51% of leukoplakia, and 37% of these patients developed HNSCC compared with only 6% of leukoplakia lacking LOH in these regions [100]. When there is additional LOH at the 4q, 8p, 11q, 13q and 17p loci, the increased risk of malignant transformation increases to 33-fold [101].

The EPOC trial, while not finding efficacy for treatment, did validate LOH as a marker of oral cancer risk. This trial investigated whether the EGRF inhibitor erlotinib could reduce malignant transformation in high-risk OPMLs, defined as OPMLs displaying LOH at either 3p14 or 9p21 with a history of oral cancer or LOH at 3p14 and/or 9p21 plus one other chromosomal site if there was no history of oral cancer [91]. In this cohort, individuals with LOH negative OPML were significantly less likely to progress to cancer, with 13% developing cancer over 3 years, compared with 26% of individuals with LOH positive OPMLs [91].

Modifiers of Expression: lncRNA and miRNA

Alterations to protein coding sequences encompass only part of the complexity in cancer. Cancer-related changes in microRNA (miRNA), long non-coding RNA (lncRNA), short nucleolar RNA (snoRNA) and epigenetic modification have been demonstrated to influence oral carcinogenesis and malignant transformation [102]. miRNAs are short noncoding RNAs 21–23 nucleotides in length that regulate the expression of 30–60% of protein-coding genes and are also able to influence epigenetic remodelling [103]. miRNAs have been demonstrated to act as oncogenes (e.g. miR21) or tumour suppressor genes by influencing the expression of tumour suppressor or oncogenic proteins.

There are several lines of evidence suggesting that miRNAs can be implicated in oral malignant transformation. miR21, miR181b and miR345 have been found to be positive markers of malignant transformation [104]. miR21 is a known oncogenic miRNA that is present in a number of cancer types and has been linked to poor patient prognosis in tongue cancer [105]. The study in tongue cancer also identified miR7 as another candidate oncogene and miR375 and miR-494 as candidate tumour suppressors [105]. Expression of these miRNAs was then assessed in progressive versus non-progressive OPMLs (5-year follow-up), and low levels of miR375 were predictive for malignant transformation [106]. It is thought that miR375 may affect the expression of Survivin via the transcription factor KLF5, thus influencing apoptosis and proliferation and potentially influence invasiveness [107]. Salivary levels of miR31 were recently shown to be an independent risk factor for the progression of OPMLs [108]. Screening of miRNAs in saliva has tremendous promise as a non-invasive strategy for monitoring lesions but has not yet been clinically validated.

lncRNAs are non-coding RNAs of more than 200 nucleotides in length. They are known to silence miRNAs and modulate the expression and cellular localisation of proteins [109, 110]. Several lncRNAs have been implicated in cancer. A study in OSCC found that the lncRNA HOTAIR was upregulated (metastasis) and *GAS5* (growth arrest-specific transcript 5) and *MEG-3* (maternally expressed gene 3, tumour suppressor) were downregulated compared with normal tissue [111]. This study also identified two novel tumour-suppressive lncRNAs that were downregulated in OSCC—*lnc-LCE5A-1* and *lnc-KCTD6-3*. These were able to reduce proliferation and migration as well as gene expression associated with stem cells and the epithelial-mesenchymal transition (EMT) when overexpressed in HNSCC cell

lines. A recent discovery study identified 728 lncRNAs that were expressed differently in HNSCC versus normal adjacent tissues, including some that had been previously implicated in cancer [112]. This study provides a pool of differentially expressed lncRNA that can be used to further investigate the use of lncRNA as biomarkers in OSCC.

Gene and Protein Biomarkers by Hallmark of Cancer Characteristics

There is a plethora of gene and protein biomarkers that have the potential to identify and/or predict malignant transformation. They have been divided into functional groups by cancer hallmarks and are summarised in Fig. 3.2.

Sustained Proliferative Signalling and Evasion of Growth Suppressors and Apoptosis

In normal cells, signalling, typically involving kinase receptor pathways and growth factors, regulates progression through the cell cycle and cell growth. Cancer cells are able to circumvent this control and thus maintain proliferation in a number of ways. This may involve the direct production of growth factors by cancer cells, the production of signals stimulating surrounding cells to produce growth factors, the overexpression of receptor proteins at the cell surface rendering the cell hyperresponsive to proliferative signalling or the activation of downstream mediators of the signalling pathway [18]. In oral cancer, the signalling molecules EGFR, FGFR, MET, PIK3CK and CCND1 and members of the Wnt pathway (AJUBA, FAT1 and NOTCH1) are important in maintaining the proliferative signalling characteristic of malignant cells. Cancerous cells must also avoid the endogenous suppression of proliferative signalling that operates in normal cells. Typically, this control over cell growth is orchestrated by tumour suppressor proteins that regulate the switch between proliferation and apoptosis/senescence. Tumour suppressor proteins may also induce apoptosis, for example, TP53 acts by inducing apoptosis when DNA damage and chromosomal abnormalities become too great [113]. Apoptosis involves balancing of the pro- and anti-apoptotic members of the B-cell lymphoma 2 (Bcl2) family. TP53 is one of the classic tumour suppressor proteins and is mutated in 69.8% of HNSCC in TCGA cohort and 42% of samples in the Pan Cancer cohort [47, 114]. Interestingly, elephants, which have a low rate of cancer compared to other mammals, were recently found to carry up to 20 copies of p53, and elephant cells show increased rates of apoptosis after exposure to a mutagenic stimuli [44].

Mutations in the epidermal growth factor receptor (EGFR) are present in 15% of HPV-negative and 8% of HPV-positive HNSCC [47]. Most HNSCC display high EGFR expression compared with normal tissue and high expression of EGFR as well as its ligand transforming growth factor-alpha is associated with poor progno-

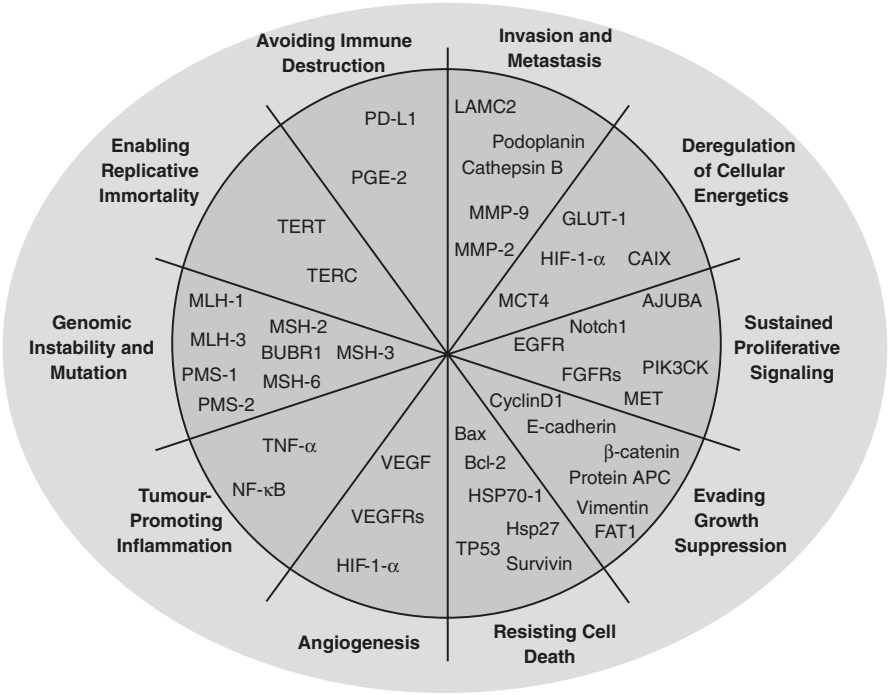

Fig. 3.2 Gene and protein biomarkers divided by hallmark of cancer characteristic. The identification of sensitive and accurate biomarkers of oral malignant transformation is a powerful tool in the early identification of OSCC. The figure displays a collection of biomarkers, divided into their hallmarks of cancer characteristics that have been identified as potential biomarkers in OPML and/or OSCC. Full names and abbreviations are provided below. Adenomatous polyposis coli protein (**Protein APC**); LIM domain-containing protein ajuba (**AJUBA**); apoptosis regulator BAX (**Bax**), also known as Bcl-2-like protein 4 (Bcl2-L-4); apoptosis regulator Bcl-2 (**Bcl-2**); MAD3/BUB1-related protein kinase (**BUBR1**), also known as mitotic checkpoint serine/threonine-protein kinase BUB1 beta (BUB1-β); catenin beta-1 (**β-catenin**); carbonic anhydrase 9 (**CAIX** or CA9); **Cathepsin B**; G1/S-specific cyclin-D1 (**Cyclin D1**); epithelial cadherin (**E-cadherin**), also known as Cadherin 1; epidermal growth factor receptor (**EGFR**); protocadherin Fat 1 (**FAT1**); fibroblast growth factor receptors 1, 2 and 3 (**FGFR-1**, **FGFR-2** and **FGFR-3**); glucose transporter type 1 (**GLUT-1**), also known as solute carrier family 2, facilitated glucose transporter member 1 (SLC2A1); heat shock 70 kDa protein 1 (**HSP70-1**), also known as heat shock 70 kDa protein 1A; heat-shock protein beta-1 (**HspB1**), also known as heat shock 27 kDa protein (**Hsp27**); hypoxia-inducible factor 1-alpha (**HIF-1-α**); laminin subunit gamma 2 (**LAMC2**); monocarboxylate transporter 4 (**MCT4**), also known as solute carrier family 16 member 3 (SLC16A3); tyrosine-protein kinase Met (**MET**), also known as hepatocyte growth factor receptor (HGF receptor); matrix metalloprotease 2 and 9 (**MMP-2** and **MMP-9**); MutL protein homolog 1 and 3 (**MLH1** and **MLH3**), also known as DNA mismatch repair proteins Mlh1 and Mlh3; MutS protein homolog 2, 3 and 6 (**MSH2**, **MSH3** and **MSH6**), also known as DNA mismatch repair protein Msh2 (hMSH2, hMSH3 and hMSH6); neurogenic locus notch homolog protein 1 (**Notch 1**); nuclear factor NF-kappa-B p65 subunit (**NF-κB**), also known as transcription factor p65; apoptosis inhibitor survivin (**Survivin**), also known as baculoviral IAP repeat-containing protein 5 (BIRC5); telomerase RNA template component (**TERC**); telomerase reverse transcriptase (**TERT**); tumour necrosis factor alpha (**TNF-α**); cellular tumour antigen p53 (**TP53**); programmed cell death 1 ligand 1 (**PD-L1**); prostaglandin E_2 (**PGE-2**), produced by the protein cyclooxygenase 1 or 2 (COX-1, COX-2); **Podoplanin**; p110α catalytic subunit of phosphinositol-3-kinase (**PIK3CA**); post-meiotic segregation increased protein homolog 1 and 2 (**PMS1** and **PMS2**); vascular epidermal growth factor a (**VEGF-A** or **VEGF**); **Vimentin**; vascular endothelial growth factor receptor 1, 2 and 3 (**VEGFR-1**, **VEGFR-2** and **VEGF-3**)

sis [115]. A recent study found that abnormal EGRF gene copy number was a positive predictor for malignant transformation of OPMD [116]. The EPOC trial also found that increased EGRF gene copy number was associated with reduced cancer-free survival in OPMLs and correlated with LOH [91].

Fibroblast growth factor receptors (FGFRs) have diverse functions. Ligand binding triggers downstream signalling that influences differentiation, proliferation and angiogenesis. Gain of function mutations in receptors and ligands have been reported in other cancer types (reviewed in [117]). FGFR1 displays genetic alteration in 10% of HPV-negative HNSCC and FGFRs 2, 3 and 4 in 2% or less. Eleven percentage of HPV-positive HNSCC display alterations in FGFR3 and 3% mutations in FGFR3 [47]. Immunohistochemical staining of FGFR-2 and its ligand FGF-2 was recently performed in OPML and OSCC samples, and their expression found to be a positive predictor of malignant transformation [118]. FGFR-3 expression was present in 48% and FGFR-4 expression in 41% of OSCCs [119, 120].

MET (hepatocyte growth factor receptor) is a proto-oncogene that signals from the extracellular matrix to the cytoplasm. Once it has bound to hepatocyte growth factor, it has pro-survival properties, including roles in migration, invasion and angiogenesis in cancer. MET or its ligand is expressed in approximately 80% of HNSCC, despite only being mutated or amplified in a relatively low number of HNSCCs in TCGA [88, 121].

CCND1 is the gene coding for the cyclin D1 protein. It is the regulatory component of the CDK4/cyclinD1 complex which regulates the cell cycle from G1/S transition. CyclinD1 modulates expression of CDK4 kinase (GeneCards). Twenty-four to 48% of dysplastic OPML had alterations in CCND1 [122]. TCGA found that the 11q amplicon, which contains the CCND1 gene, is frequently altered in HPV-positive HNSCC. Approximately 20% of HNSCC display mutations in CCND1 [114]. Expression of cyclinD1 measured by IHC correlated with malignant progression of leukoplakia and erythroplakia [123, 124]. CyclinD1 is believed to be upregulated early in oral carcinogenesis and can be detected in saliva in individuals with oral cancer [50].

The PIK3CK gene encodes the p110 alpha protein, which is the catalytic subunit of phosphatidylinositol 3-kinase (PI3K). PI3K is part of the AKT signalling pathway, which regulates cell proliferation, migration and survival. In oral cancer, PIK3CK is an oncogene, and mutations in PIK3CK are common, particularly in HPV-positive tumours. Approximately 21% of OSCCs display alterations in PIK3CA, and mutations activating PIK3CA were prevalent in a sub-group of OSCCs that displayed improved clinical outcomes [47, 114].

Genes of the Wnt pathway including AJUBA, FAT1 and Notch1 are important in regulating cellular proliferation and are frequently mutated in oral cancer with Notch1 mutations present in 19.3% of HNSCC [114]. Notch1 mutations are also present in leukoplakia, and around 60% of these mutations are also present in OSCC, suggesting that Notch1 mutation is an early event in carcinogenesis [125]. Inactivation of AJUBA, FAT1 and Notch1 causes deregulation of cellular polarity and differentiation and may contribute to malignant transformation.

Other members of the Wnt signalling pathway include E-cadherin, β-catenin, APC and Vimentin, which have shown to be potential markers of malignant transformation [126] and LGR5 which shows increasing expression from OED to OSCC [127].

The cyclin-dependent kinase inhibitor 2A (CDKN2A) gene codes for the p16 tumour suppressor, as well as producing several splice variants. It acts by regulating the cell cycle and is a tumour suppressor. CDKN2A is mutated in 21.3% of HNSCC [114]. In HPV-negative HNSCC, p16 expression is reduced favouring cellular proliferation [128, 129]. Infection of the oral mucosa with high-risk HPV induces overexpression of p16 in OPML and OSCC so it can be utilised as a surrogate biomarker for HPV infection, although this can lead to false positives if used alone [128–130].

Heat-shock proteins are expressed in response to stress and may inhibit apoptosis. HSP70 and HSP27 can act as markers of epithelial dysplasia in leukoplakia [131]. Apoptotic pathway members Bcl-2, Bax and Survivin have been shown to display altered expression in oral cancer and precancer [132, 133].

Replicative Immortality

Normal cells can only go through a limited number of cycles of cell division before they enter a state of senescence or crisis and subsequent apoptosis. These processes are mediated via telomeres, which wrap around the ends of chromosomes, protecting them from damage. Each cycle of cell division shortens the telomeres until they are no longer able to protect the chromosome from damage. Occasionally, cells may emerge from crisis with the capacity to undergo unlimited proliferation and are termed immortalised. Telomerase is a ribonucleoprotein which consists of two components: telomerase reverse transcriptase (TERT), the enzyme component and telomerase RNA component (TERC), which acts as a template for the telomere repeat TTAGGG which is added to telomere ends. Immortalised cells (including cancer cells) are able to prevent telomeres from eroding and express much more telomerase (which lengthens the telomere DNA) than normal cells [134, 135]. Telomerase reverse transcriptase (TERT) is one of the protein subunits of telomerase and has both enzymatic activity and is involved in proliferative signalling via the Wnt pathway. TERT mediates the elongation of telomeres, promotes cell immortalisation and has also been shown to increase invasiveness [136]. TERT expression is not present in normal or mildly dysplastic oral mucosa but is present in moderately dysplastic tissues as well as those displaying severe dysplasia and OSCC [137]. A recent study combining morphological assessment and detection of hTERC (the RNA component of telomerase) via FISH found that acquisition of the hTERC gene predicted malignant progression in OPMLs [138]. When telomerase activation was measured in HNSCC and OPML, the levels were comparable (78% and 85%, respectively) but had increased by 25–32% compared with normal, adjacent tissue [139]. Reduced telomere length is found in tissues adjacent to CIS, and orthokeratotic leukoplakia is associated with particularly short telomeres [51, 140].

Together, these studies suggest that cellular immortalisation occurs relatively early in oral carcinogenesis.

Invasion and Metastasis

Invasion and metastasis in oral cancer are driven by the epithelial to mesenchymal transition (EMT), a developmental program that involves the conversion of epithelial cells to a mesenchymal phenotype. This phenotypic change involves a loss of cellular polarity, a switch to a spindle shape, increased motility and resistance to apoptosis. EMT is also associated with increased expression of enzymes capable of digesting extracellular matrix to facilitate invasion.

Matrix metalloproteases are a family of zinc metalloenzymes with the capacity to digest a range of components of the extracellular matrix, including gelatin, collagen, stromelysin and membrane components. MMP-2 and MMP-9 remodel extracellular matrix and are important in mediating invasion and metastasis in OSCC. In a study of tongue cancer, MMP-2 and MMP9 were found to increase expression as carcinogenesis progressed, with rare expression in normal epithelium, expression in approximately 40% of non-metastatic tongue SCC and expression in 70–80% of metastatic SCC [141]. While normally associated with the later stages of carcinogenesis, salivary levels of MMP-9 are increased in OPMLs compared with healthy controls and then further increased in OSCCs [142]. A recent meta-analysis concluded that MMP-9 was a viable biomarker to predict malignant progression [56].

Podoplanin (coded by the PDPN gene) is a transmembrane protein that is upregulated during epithelial-mesenchymal transition and has roles in migration in many human cancers [143]. Podoplanin is a useful marker of malignant transformation in erythroplakia [144], and a recent paper has suggested that the risk of transformation in OPMLs is enhanced threefold if podoplanin is co-expressed with ELAV like RNA-binding protein 1 (HuR) [145]. Laminin subunit gamma 2 (LAMC2) is an extracellular matrix glycoprotein and a contributor to the breakdown of the basement membrane in oral cancer. When expressed in leukoplakia, LAMC2 predicts imminent malignant transformation [146]. Cathepsin D and B forms have also been correlated with invasion and metastasis in OSCC [147].

Avoiding Immune Destruction

Patients with HNSCC display immune suppression with reductions in antigen presentation, reduced lymphocyte counts and inhibited NK cell activity [97]. Evidence for this includes cases of human-associated transplant cancer, where cancer develops in immunosuppressed recipients when no longer held in check by the healthy immune system of the donors [148].

Tumour-associated macrophages (TAMs) have a demonstrated role in cancer with the M2 phenotype considered to be pro-inflammatory and tumour promoting and the M1 phenotype protective. Premalignant lesions have been shown to contain

M1 TAMs and OSCC primarily the M2 phenotypes [149, 150]. This suggests that TAM phenotype can be used as a marker of malignant transformation. IL-37 acts to suppress the innate immune system and could provide a potential marker for malignant transformation in leukoplakia as there was a correlation between the degree of dysplasia and IL-37 protein expression [151].

In the healthy immune system, CD8+ T cells and CD4+ helper T (Th)1 cells inhibit cancer formation by secreting interferon gamma (IFN-γ) and cytotoxins [126]. A recent study provided evidence that the presence of CD3+ T cells may protect against malignant transformation. The dysplastic leukoplakia that progressed to OSCC had significantly less CD3+ T cells than those that did not [152].

OPMLs have been shown to display increasing immune cell infiltration as they progress through grades of dysplasia and into carcinoma [150]. Ohman et al. demonstrated increased numbers of Langerhans cells and T cells in dysplastic cell compartments and OSCC [153]. OPMLs may also start to induce inflammation and modulate the immune system before they undergo malignant transformation. In a mouse model, cells derived from premalignant oral lesions secreted substances that induced T-cell activation and expression of IL-2 receptor [102]. Programmed death 1 (PD-1) and its receptor (PD-1R) have been implicated in tumour escape and are expressed in premalignant as well as malignant tissues [154]. A recent immunohistochemical study found that 29% of OSCC lesions express PD-L1 and 83% contain PD-1 positive lymphocytes [155].

Genomic Instability and Mutation

Genomic rearrangements and mutations have been well characterised in OSCC and to a lesser extent in OPMLs. There are a number of systems that maintain genomic stability in normal cells, including the DNA mismatch repair system and the mitotic spindle checkpoint.

The DNA mismatch repair system in humans consists of MutL protein homolog 1 and 3 (MSH1 and MSH3), MutS protein homolog 2, 3 and 6 (MSH2, MSH3 and MSH6) and post-meiotic segregation increased 1 and 2 (PMS1 and PMS2), and they work sequentially to repair DNA mismatches [3]. MSH2 heterodimerises with MSH6 to form MutS-alpha, the predominant form of MutS in humans, which recognises mismatches. MLH1 and PMS2 heterodimerise to form MutL-alpha, again the primary form in humans, which links MutS-mediated mismatch repair with downstream activators [52]. Deficiencies in this system have been clearly implicated in non-hereditary colon cancer [156], but there is evidence that these are also important in OPML and OSCC.

OLP display decreased protein expression of MSH2 in comparison to normal tissues, and MLH1 protein expression can be correlated with the degree of dysplasia in leukoplakia [157]. A recent study assessed protein expression of all the major MMR components across the spectrum of OED and OSCC and found that MLH1, PMS2 and MSH2 were reduced in malignant and premalignant tissues compared with normal [158]. The dimerisation components (i.e. MSH2/MSH6 and MLH1/

PMS2) correlated with each other, and the specific loss of MSH6 in the basal layers was shown to be a useful diagnostic marker for carcinoma in situ [159].

BUBR1, one of two putative vertebrate homologs of the yeast spindle checkpoint protein BUB1, has two important roles in mitosis, with the C-terminal kinase domain regulating chromosome movement and the N-terminus involved in checkpoint, pausing anaphase until all chromosomes are properly attached to the mitotic spindle [160]. BUBR1 is overexpressed in a subset of OSCC cases [161] and promotes cellular mobility and invasion via MMPs in OSCC cell lines [162]. Immunohistochemical evidence suggests that BUBR1 is expressed in premalignant lesions and may act as a biomarker for oral carcinogenesis [63].

Conclusion

OSCC is frequently identified at an advanced stage, which limits treatment options and leads to increased patient mortality and morbidity. A better understanding of the molecular changes that drive oral carcinogenesis has the potential to deliver improved screening, treatment options and patient outcomes.

Oral carcinogenesis can be understood using clinical, histopathological and molecular models, which describe the physical manifestation of the lesion, the alterations in tissue architecture and cell cytology within the lesion and the genetic alterations leading to dysfunctional protein expression/function within the cells of the lesion. Our understanding of oral carcinogenesis has been significantly enhanced by the development of animal models which utilise carcinogenic agents applied to rats, mice or hamsters and mimic the development of human oral cancer. These, along with population-based studies, have been useful in unravelling the impact of various risk factors on the development of oral cancer, including smoking and alcohol consumption. Genetic conditions that predispose individuals to the development of cancer have also provided insight into oral carcinogenesis, particularly the importance of DNA repair systems in protecting against cancer. Large-scale sequencing efforts have provided a wealth of information about the genetic alterations that occur in cancerous cells and allowed for a more meaningful segregation of cancer subtypes and personalisation of treatment.

The development of oral cancer can be understood as the acquisition of mutations that allow the development of cancer hallmarks or properties that allow cancer cells to survive, proliferate and metastasise. Biomarkers are measurable characteristics that can be used to identify OSCC and predict which OPML are likely to transform to cancer. These include markers of genomic instability, the presence of expression modifiers such as miRNA and a range of gene and protein markers. We have divided the gene and protein markers into groupings by functional capability and assessed their value as markers of current or future malignant transformation. Changes in protein expression of many of these biomarkers have been identified in OPMLs, suggesting that they have potential utility as predictors of malignant transformation, although much more work is required to realise these in clinical practice.

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