Chapter 2
The Molecular Basis of Carcinogenesis

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In this chapter, we will discuss the molecular basis of carcinogenesis. *First understand, and then treat!* Better treatment options for cancer and preventive approaches for potentially malignant lesions can be achieved only if the pathobiology of the disease is well understood. We have witnessed a shift in the therapeutic approaches to cancer, from "universal" therapies applied to several different tumour types to tailored and personalized treatment. Each tumour/lesion is unique. As the understanding of malignant transformation and carcinogenesis requires knowledge of molecular and tumour biology, we aim to discuss carcinogenesis initially in a broader context before discussing the effects of carcinogens on the aetiology of potentially malignant oral lesions.

Starting from the Beginning: Useful Concepts

Carcinogenesis Theories and Field Cancerization in Oral Epithelium

How does cancer arise? Is it merely a result of the accumulation of mutations over time? Is cancer a disease of the cell, or is it a disease of the tissue and of cell signalling in the microenvironment? There are several theories that attempt to explain the process of carcinogenesis by incorporating evidence and developing models [1].

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Among these theories are coherent non-exclusive models of carcinogenesis that focus on the biological changes in the epithelium alone, whereas other models also take the changes in the stroma into account. By far, the most widely disseminated carcinogenesis theory is the “somatic mutation theory” (SMT), which is based on the assumption that cancer is derived from a single somatic cell that accumulates DNA mutations. The SMT focuses on molecular changes in the epithelium. On the other hand, the “tissue organization field theory” (TOFT) considers carcinogenesis as a problem of tissue organization, highlighting the importance of stroma in the process of carcinoma formation [2]. There are strengths and weaknesses in both models, and they are not mutually exclusive in some areas; however, the TOFT carcinogenesis model has gained acceptance recently, as more scientific evidence has strengthened the importance of the microenvironment in tumour formation, demonstrating that cancer is a disease of the tissue and not simply a cellular disease.

Regardless of the carcinogenesis model chosen to explain how normal cells become cancer cells, one needs to consider basic concepts in human molecular genetics, as clinical and histopathological morphological changes are accompanied by molecular changes in tissues. Slaughter proposed in 1953 the field cancerization process in oral stratified squamous epithelium, showing that clinically normal tissue surrounding oral squamous cell carcinoma (OSCC) already harboured histopathological changes [3]. Interestingly, once the structure of DNA was solved, the field cancerization concept evolved and was updated, and it became known that clinical and morphological normal tissues surrounding OSCC had already incorporated molecular changes [4] (Fig. 2.1). An understanding of this concept is fundamental for those studying/treating OSCC and oral leukoplakia. The field cancerization in oral mucosa can be as large as 7 cm [5], which means that by removing an oral leukoplakia lesion, one cannot remove all cells that have been molecularly altered. This knowledge is also fundamental when interpreting research studies whose

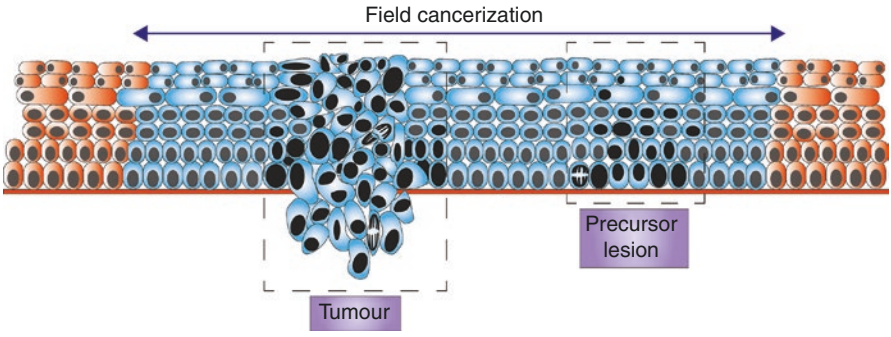

Fig. 2.1 Field cancerization. An area of epithelial cells harbouring molecular alterations (blue cells). A molecularly altered field can occur with normal histology, and in this figure we can observe a precursor lesion (oral leukoplakia) and an OSCC occurring in a same field cancerization

normal control reference tissues are “normal” tissues adjacent to the OSCC/oral leukoplakia.

Every pathology textbook describes the initiation and progression of cancer from a “clonal evolution” perspective. During clonal evolution, gradualism is assumed to occur, i.e. phenotypic features in cancers are believed to develop at a slow and continuous rate. According to clonal evolution, tumours are monoclonal, as they are derived from a single somatic cell, followed by the development of a neoplasm with cellular heterogeneity as a result of continued mutagenesis (we will discuss this topic in another section). When the tumour mass is established, clonal selection of the most well-adapted cells occurs, and the new, more fit clones rise to dominance and replace the entire population. This theory became the standard model of carcinogenesis and continues to spread, primarily because it is a simple and uncomplicated manner to explain a complex process. However, in this clonal evolution theory, even the definition of a “clone” is not unequivocal and straightforward and can be interpreted in more than one way [4]. Another caveat is that if cancers evolve linearly with time (gradualism), the malignant transformation of potentially malignant lesions, such as oral leukoplakia and Barrett’s oesophagus, should be predicted easily [6]. However, this phenomenon is not what happens in the clinic, as it is impossible to predict which “premalignant” lesions will evolve to become cancer.

Genetic progression models for oral leukoplakia have been proposed based on the somatic mutation carcinogenesis theory and on clonal evolution [5]. A monoclonal origin from OSCC associated with oral leukoplakia has been suggested, assuming that the carcinoma originated in the adjacent oral leukoplakia [7]. This hypothesis, however, is speculative, as retrospective studies using only the biopsy tissue from the excision of an OSCC lesion (including the adjacent oral dysplasia area) might not represent a true malignant transformation. OSCC is not always preceded by oral leukoplakia. To add a further layer of complexity to this subject, technological developments in genome analysis and mathematical and bioinformatics techniques have shown that the phenomena of punctuated and neutral evolution occurs during tumour evolution [6], and clonal evolution theory and gradualism fail to explain these findings. During the cancer evolutionary process, the genome is shaped not only by random mutations and non-random selection but also by random drift [4]. Both drift and selection change the frequency of alleles in a population, drift by random processes and selection based on *fitness*. Neutral evolution is defined as when selection is not operating and only the stochastic process of random mutations and drift occur. While random mutations and non-random selection have been the focus of several tumour evolution studies, random drift remains poorly understood, which does not allow for a complete understanding of how tumours evolve. A better understanding is yet to be obtained.

In the following sections, we will review briefly some basic concepts in human molecular biology. These definitions will help in following the discussions on cancer molecular pathogenesis.

DNA, RNA, Noncoding RNA, and Protein

The human genome is composed of DNA that contains approximately three billion base pairs distributed among 23 chromosome pairs (22 autosomal chromosomes and one sex chromosome). DNA molecules carry genetic information inside the cells and are composed of a double strand of linear polymers of nucleotides. DNA is packed inside the chromosomes in association with histone proteins, forming the nucleosomes. Each nucleosome consists of eight histone proteins around which DNA is wrapped [8], as shown in Fig. 2.2.

DNA is composed of the nucleotides adenine (A), cytosine (C), guanine (G), and thymine (T). It is organized into functional and physical units of heredity called genes. Genes have introns (regions which do not code for proteins) and exons (protein-coding sequences). The genetic DNA code is transcribed into mRNA, which is translated into proteins in that three nucleotides (codon) code for a specific amino acid in the protein (or are stop codons) [8].

Less than 2% of the human genome encodes proteins! Genetic sequencing of these protein-coding regions of the human genome is referred to as *whole-exome sequencing* (WES), and it is currently being used in biomedical research as well as

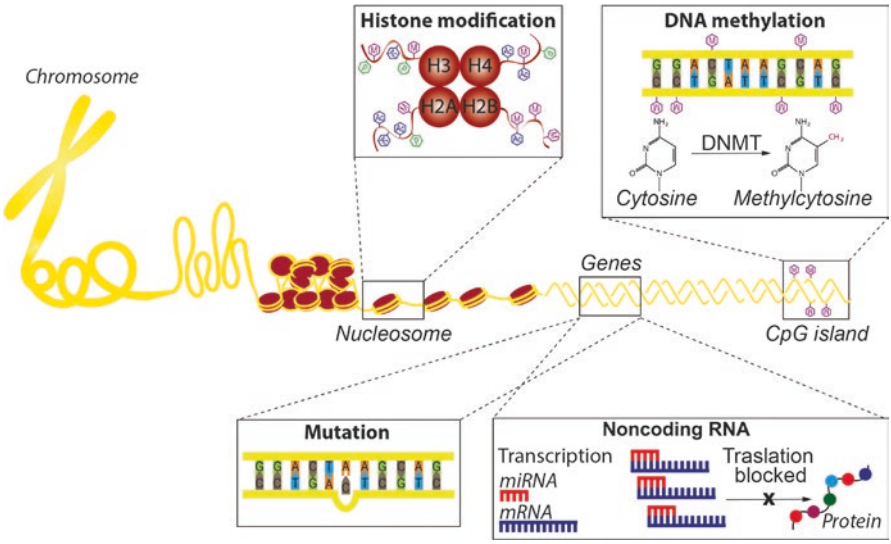

Fig. 2.2 DNA organization and carcinogenesis-related alterations. DNA is packaged in chromosomes forming complexes with histones. These complexes are the nucleosomes, and each nucleosome consists of eight histone proteins around which DNA is wrapped. Several alterations at nucleosome and nucleotide levels occur in carcinogenesis. The histone N-terminal tails modulate nucleosome structure and function and can suffer modifications, which include changes in their methylation and acetylation profiles. At nucleotide level, DNA mutations cause inactivation of tumour suppressor genes or activation of oncogenes. Gene expression levels can be altered by modifications in DNA methylation profiles (repressing transcription) or by ncRNA activity (repressing translation)

in the diagnosis of human diseases. Surprisingly, approximately 75% of the genome is transcribed into RNAs, including RNAs that have no protein-coding potential (noncoding RNAs) [9, 10]. Noncoding RNAs (ncRNAs) <200 nt are classified as small ncRNAs. Micro-RNAs (miRNAs) are a category of small ncRNAs. Conversely, ncRNAs >200 nt are classified as long ncRNAs (lncRNAs). While miRNAs are primarily involved in "silencing" gene expression (by targeting mRNAs) (Fig. 2.2), lncRNAs, which are more abundant than miRNAs in the human genome, exhibit a greater variety of functions in the regulation of gene expression [9].

miRNAs have been extensively studied in OSCC, and lncRNAs are in the process of being better characterized in such tumours [11, 12]. miRNA profiling in progressive and nonprogressive oral leukoplakias has shown that miR-21, miR-181b, and miR-345 increased expression in oral leukoplakias that progress to OSCC [13]. Additionally, higher expression levels of these miRNAs were found to be associated with cytological and histopathological parameters used to grade dysplasia, including an increased nuclear/cytoplasmic ratio and the presence of abnormally superficial mitosis [14]. LncRNA expression in oral premalignant lesions has been reported [15] but requires additional characterization and functional studies to better reveal the roles of such ncRNAs in the biology of these lesions.

Mutation and Genetic Variation

"There is no single sequence of the human genome." There are approximately three million sequence variations between any two unrelated persons, most of which do not have biological importance and do not contribute to physiological differences but do give rise to diversity between individuals.

Genetic variations that occur at a measurable frequency in the population are termed polymorphisms. A strict definition of a genetic polymorphism is variation present at a frequency ≥1% in the population. When a polymorphism is characterized by the substitution of a single nucleotide (e.g. the substitution of a C<T at a given position), it is defined as a single nucleotide polymorphism (SNP). Thousands of SNPs have been described, and there is a database of SNPs (and other short genetic variations) that can be accessed at https://www.ncbi.nlm.nih.gov/snp.

A mutation occurring in an exon (i.e. DNA that codes for proteins) can result in a change from one amino acid to another (missense mutation), a change that codes for a termination signal/stop (nonsense mutation), or no change in the amino acid (silent mutation). Mutations characterized by an insertion or deletion of one to a few nucleotides are called indels.

When DNA mutations are found in a given tumour, but not in peripheral blood/normal matching tissue, the mutation is considered a *somatic* mutation that originated in the tumour. However, if the mutation is also detected in normal constitutive DNA, it is classified as a *germline* mutation. An example of a germline mutation that predisposes individuals to cancer is the mutation in the *TP53* gene in Li-Fraumeni syndrome. However, the majority of tumours arise from somatic mutations and are

considered sporadic rather than familial tumours. Somatic mosaicism may occur, and a germline mutation cannot be detected in every constitutive normal cell; however, we will not discuss this topic in this review.

With the advances in next-generation sequencing (NGS) technology, the characterization of somatic genomic alterations in head and neck squamous cell carcinoma (HNSCC) is beginning to emerge. Recently, The Cancer Genome Atlas (TCGA) has profiled 279 cases of HNSCC by undertaking a comprehensive multiplatform characterization [16]. Similar to lung cancer and melanomas, HNSCC exhibits a high incidence of somatic mutations, which is consistent with its chronic exposure to mutagenic factors (tobacco smoking) [17]. Genes frequently mutated in HNSCC include *TP53*, *NOTCH1*, *HRAS*, *PIK3CA*, and *CDKN2A* [16]. *NOTCH1* gene mutations have been reported in a high proportion of oral leukoplakias and in OSCC, which raises the possibility of these mutations being important OSCC progression drivers [18].

Cell Cycle Differences Between Normal and Cancer Cells

Cell division occurs through sequential events that drive the progression from one cell cycle stage to the next, and it is altered in cancer cells [19]. The cell cycle is divided into two major phases, which are interphase and mitotic (M) phase. Interphase is subdivided into G1, S, and G2 phases. During G1, the cell grows and copies organelles; while in the S phase, the cell duplicates the DNA in the nucleus and in the centrosome. When the cell enters G2, it grows, synthetizes proteins and organelles, and prepares for mitosis. During the M phase, the cell separates its DNA and cytoplasm, leading to the formation of two cells.

Normal cells move through the cell cycle in a regulated manner, ensuring that they only divide when their DNA is not damaged and when there is room for more cells in the given tissue. The most important checkpoints that regulate the cell cycle are at the G1/S transition, the G2/M transition, and in the M phase. The cell cycle may be interrupted at any of these checkpoints so that the DNA can be repaired or that the cell can be eliminated by apoptosis.

Cyclins are one of the core cell cycle regulator proteins. Cyclins form complexes with cyclin-dependent kinases (CDKs), which in turn phosphorylate target proteins. There are several different cyclins, and the levels of each cyclin vary across the cell cycle, usually increasing only at the stage where they are required. Genetic mutations affecting cyclin or CDK genes can result in uncontrolled cell cycle progression. Cyclin D1, for example, is overexpressed in a variety of human cancers, including OSCC [20]. Conversely, there are CDK inhibitors that negatively control the cell cycle, including several different proteins such as p21, p16, p27, and p57. These proteins are frequently mutated or silenced by other mechanisms such as DNA methylation in human cancers. As CDKs play a central role in controlling cell cycle pathways, the development of therapeutic approaches to inhibit their kinase activity in cancer cells is currently in progress [21].

Alterations in the cell cycle include, but are not restricted to, genetic mutations (we will discuss this later in this chapter) and confer tumour cells with growth and survival advantages. While the normal cell cycle is regulated by proto-oncogenes, tumour suppressor genes, apoptosis genes, as well as DNA damage repair genes, in human neoplasia, these genes are usually dysregulated.

Oncogenes and Tumour Suppressor Genes

Oncogenes and tumour suppressor genes control cellular proliferation. An oncogene is a mutated form of a normal cellular gene referred to as a proto-oncogene. Proto-oncogenes are genes that positively regulate the cell cycle, and when they are over-activated by mutations, they are called oncogenes. This transformation of a proto-oncogene to an oncogene involves changes in protein amino acids, which can alter the protein structure. The mutations that convert proto-oncogenes to oncogenic alleles are named *activating mutations* to reflect "the gain of function". Additionally, proto-oncogene activation also can occur by gene amplification, in which extra gene copies are accumulated in the cell, resulting in extra protein production, or by chromosomal translocation (involving different mechanisms) [22].

Tumour suppressor genes are negative regulators of the cell cycle, and their functions are usually impaired in cancer. In contrast to proto-oncogene activating mutations, tumour suppressor genes usually harbour *loss-of-function* mutations with proteins that become functionally inactivated in cancer. Tumour suppressor genes normally control processes such as maintenance of genetic integrity, differentiation, cell-cell interactions, progression of the cell cycle, and apoptosis. Therefore, inactivation of tumour suppressor genes contributes to the disturbance of tissue homeostasis [23]. The most extensively studied tumour suppressor gene in human cancer is the *TP53* gene [24]. *TP53* prevents neoplastic transformation by temporarily or permanently activating the interruption of the cell cycle or by signalling cell death, and it is mutated in approximately half of all human cancer cases, including OSCC [16]. *TP53* is more frequently inactivated by small alterations, primarily by single nucleotide point mutations, and they occur at a higher frequency in hot spots that interfere with the functions of the encoded protein, which correspond to exons 5–8 of the gene.

Genetic Instability

Cancer cells commonly harbour defects in the mechanisms by which the genome is replicated and repaired and by which chromosomes are segregated during the cell cycle. These defects result in a higher rate of genetic alterations in cancer cells compared to normal cells and are less stable genetically than the surrounding normal tissue [25]. This genetic instability accelerates the occurrence of subsequent genetic

alterations; however, while genetic instability is a defect in a process, genetic alterations are stochastic events that do not necessarily indicate or cause genetic instability.

Genetic instability can be categorized into the following two major groups: instability at the nucleotide level and instability at the chromosomal level (chromosomal instability, CIN). Nucleotide-level instability includes deletions, insertions, and base substitution, while CIN refers to an increased rate of chromosome gains and losses, involving chromosomal missegregation due to mitotic errors [26]. A loss of specific chromosomal regions at constitutive heterozygous loci (loss of heterozygosity, LOH) that spans tumour suppressor genes has been reported to be a good predictor of malignant transformation of oral leukoplakia. Oral leukoplakias with LOH at chromosome regions 3p and/or 9p exhibited a markedly higher chance of malignant transformation compared to cases with 3p and 9p retention [27]. CIN involves cytogenetic changes that lead to changes in chromosome copy number, i.e. aneuploidy. Human cells contain 23 pairs of chromosomes and are diploid. A cell that has a number of chromosomes that is not a multiple of the haploid number is *aneuploid*. Aneuploid cells not only have a numerical abnormality but also commonly have chromosomal structural aberrations [26]. Aneuploidy occurs in a high proportion of solid human tumours, including OSCC [28]. In addition, as some OSCC arise in precursor lesions (potentially malignant oral disorders, including oral leukoplakia) and in preneoplastic epithelium, they can exhibit aneuploidy [29], and several studies have examined the possibility that aneuploidy indicates a risk of malignant transformation [30, 31]. Sperandio and co-workers [30] published a large series of DNA ploidy investigations in oral dysplasia, including 273 patients (32 with malignant transformation), for 5–15 years and demonstrated a positive predictive value for the malignant transformation by DNA aneuploidy of 38.5% [30]. In their study, the DNA ploidy status appeared to be correlated with epithelial dysplasia, and by combining both (ploidy status and dysplasia grading), the predictive value was higher than by using either technique alone. The utility of using DNA ploidy to predict the risk of oral dysplasia malignant transformations can vary according to the technique used, i.e. by flow or image cytometry [32].

While aneuploidy is a hallmark of several solid tumours, others do not show aneuploidy but rather exhibit defects in DNA repair. In a normal cell, DNA sequence errors arise as a result of mutagenic effects of environmental agents. In addition, errors caused by DNA polymerase arise during cell division (i.e. an endogenous form of mutagenesis). However, normal cells contain the machinery to repair these errors, as there are more than 100 known human DNA repair genes [33].

DNA repair pathways are classified into the following three functional categories: (1) direct reversal of DNA damage, (2) excision repair of DNA damage, and (3) DNA double-strand break repair. In the first pathway, a single enzyme repair system can restore the conformation of pyrimidines after UV light damage in a relative simple light-dependent reaction. The second pathway is composed of the following three different repair systems: base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR) genes. BER proteins excise and replace

a single base and are commonly used to repair damage caused by insult to endogenous DNA (such as in response to oxidative DNA damage). NER excises oligonucleotides in response to genomic damage caused by UV exposure and involves at least 30 different proteins. MMR, the third excision repair system, preserves genomic integrity by acting in cases that involve inaccuracy in DNA replication. In the occurrence of a mutation during DNA replication, MMR recognizes and excises the mismatched nucleotide, resynthesizes DNA, and then ligates the broken strand. In addition, a direct reversal of DNA damage and excision repair of DNA damage can be repaired by a third pathway, which involves the repair of double-stranded DNA. This pathway uses a number of proteins to repair double-stranded DNA breaks (DSBs) that result from exogenous and endogenous agents, including ionizing radiation, chemical exposure, and somatic DNA recombination [33].

All of these mechanisms of DNA damage repair are interconnected and act cooperatively to maintain genome integrity. However, in cancer, these repair systems may be impaired. Mutations or loss of function of these genes may result in a reduced capacity for the correction of DNA errors, thereby predisposing the cell to genomic instability. If the functions of these genes are impaired, then the cell cannot repair the DNA, and programmed cell death can be triggered following the activation of apoptotic genes.

Evasion of Apoptosis

Tumour growth results not only from increased cell division, but it also depends on preventing cells from entering apoptosis. Neoplastic cells have the capacity to evade apoptosis by several mechanisms, enabling them to increase in number. These apoptosis-evasion mechanisms include the amplification of anti-apoptotic machinery, downregulation of the pro-apoptotic program, or both [34, 35]. There are several examples of altered regulation of genes that encode either the anti-apoptotic or pro-apoptotic Bcl-2 family in cancer. The *BCL-2* anti-apoptotic gene was first described because of its translocation in non-Hodgkin lymphomas, and it is also amplified in other tumour types [34]. Another mechanism that can lead to the overexpression of BCL-2 is the loss of micro-RNAs that repress BCL-2 gene expression, as observed in chronic lymphocytic leukaemia, in which micro-RNA 15 and 16 genes are deleted [10].

Immunotherapy and Immune Escape

The microenvironment is a critical regulator of tumour biology and can either inhibit or support malignant transformation and tumour development, growth, invasion, and metastasis. One important component of the tumour microenvironment is the immune system. Tumour cells express antigens that can mediate their

recognition by host CD8+ T cells and allow clinically detected tumours to evade antitumour immune responses.

Immunotherapy is an old concept, which has recently gained increased attention from the scientific community. These strategies are designed to alter the immune system, either by stimulating the patient's own immune system to attack cancer cells or by providing "immune system man-made components" such as proteins. Unfortunately, not all tumours respond to immunotherapy, and to increase the efficacy of immunotherapy, the immune escape mechanisms used by cancer cells must be overcome. Tumour cells can evade immune elimination by different mechanisms, such as the loss of antigenicity and/or the loss of immunogenicity, and by establishing an immunosuppressive microenvironment [36]. Immunotherapy is beginning to be explored in the oral cancer scenario, but the majority of novel immunotherapeutic strategies are currently investigational [37].

Epigenetics: Changes Beyond Genetic Sequence Changes

It is common to consider cancer a "genetic" disease. However, genetics and epigenetics cooperate in cancer development and progression. There is crosstalk between the genome and the epigenome. Genetic alterations of the epigenome contribute to cancer, and additionally, epigenetic processes can cause point mutations and disable DNA repair [38]. Epigenetics is defined as "heritable changes in gene expression that are not accompanied by changes in the DNA sequence". If we are not strict with the "heritability", noncoding RNAs can be considered epigenetic modifiers, and they have been discussed previously in this chapter. However, the most important epigenetic modifiers in cancer are DNA methylation, histone modification, and chromatin remodelling.

DNA methylation is classically associated with gene silencing, although other functions have recently been described. It occurs on cytosine, which is converted to 5-methylcytosine by the action of DNA methyltransferase (DNMT) enzymes (Fig. 2.2). Frequently, the altered C is adjacent to a G, and methylation is distributed in CpG sequences throughout the genome. CpGs are clustered in CpG islands, often at gene promoters (i.e. at the start of genes, where transcription machinery binds) (Fig. 2.2). CpG islands tend to be unmethylated, and when methylation occurs in CpG islands, it results in silencing of gene expression. DNA methylation can lead to gene silencing by different mechanisms that involve the physical impediment of transcriptional proteins binding to the gene and the indirect alteration of chromatin structure, forming heterochromatin. Heterochromatin is a compact and inactive form of chromatin. In cancers, the earliest epigenetic aberration found was a genome-wide hypomethylation [38]. Head and neck squamous cell carcinoma (HNSCC) exhibits global genomic

hypomethylation [39]. The degree of global methylation was associated with smoking history as well as with alcohol use and tumour stage in a large cohort of HNSCC samples [40].

In addition to DNA methylation, gene expression can be epigenetically modified by histone modifications, which include acetylation and methylation (Fig. 2.2). Most histone modifications occur on the N-terminal tails that protrude from the nucleosome (Fig. 2.2). Histone acetylation is universally correlated with gene activity and occurs at lysine (K) residues, and as it lacks mechanisms for mitotic heritability, it is considered a chromatin modification rather than an epigenetic modification. Histone methylation, on the other hand, can correlate either with transcriptional activity or with inactivity, and it occurs primarily at lysine (K) and arginine (A) residues [38]. Other histone modifications are less well characterized and include ubiquitination, phosphorylation, sumoylation, ADP-ribosylation, and citrullination. Very recently, impaired histone methylation (Histone H3 at K36, i.e. H3K36) was proposed to have a potential role in the development of a subset of HNSCC [41].

Interestingly, as a result of advances in next-generation sequencing, it was revealed that more than 50% of human cancers harbour mutations in chromatin organization enzymes. As tumour cells use epigenetic processes to escape from host immune responses and from chemotherapy as well, a growing number of studies are investigating drugs that target epigenomic alterations in cancer, including DNA methylation and histone modifications [42].

Intra-Tumour Heterogeneity

All of the aspects of tumour biology and molecular alterations and capabilities that have been described above must be understood in light of the "tumour heterogeneity" issue. Not all tumour cells share the same genetic and phenotypic traits, i.e. populations of tumour cells within the same tumour display remarkable variability. Intra-tumour heterogeneity is evident at the genetic and epigenetic levels as well as at the transcriptomic and proteomic levels [43, 44]. Intra-tumour heterogeneity is a phenomenon that has been known for several years, but it has recently gained more attention, as heterogeneity is a major obstacle to therapeutic success. Individual tumours may achieve resistance via several routes simultaneously, due to intra-tumour heterogeneity [45].

It is becoming increasingly evident that most, if not all, solid tumours exhibit evidence of intra-tumour heterogeneity. For some cancers, such as HNSCC and oesophageal and breast cancer, the degree of intra-tumour genetic heterogeneity is associated with a poor prognosis and a more negative clinical outcome. Of note, oral leukoplakia also shows intra-lesion heterogeneity with coexisting multiple "clones" [7].

Aetiological Factors for Oral Potentially Malignant Disorders (OPMDS) and Mechanisms of Carcinogenesis

Different aetiological factors are able to provoke genetic and epigenetic alterations in the genome (Fig. 2.3) [46]. Recent advances in sequencing technologies have deciphered the molecular signatures caused by mutagenic agents. For example, ultraviolet light (UV) and aflatoxin leave distinct patterns of DNA mutations in squamous cell carcinomas and hepatocellular carcinomas, respectively. Below, we review the most important aetiological factors currently associated with the occurrence of OSCC and oral potentially malignant disorder (OPMD).

Tobacco Smoking

Oral leukoplakia is the main oral potentially malignant disorder (OPMD). Although the association of oral leukoplakia with smoking and alcohol is well accepted in the literature, there is a lack of well-designed studies that deeply investigate this issue [47]. Systematic reviews are hampered by the heterogeneity of the studies and by changes in the oral leukoplakia concept and definition with time. The association between tobacco smoking and oral leukoplakia is based primarily on observational studies that report the disappearance of some lesions following the cessation of tobacco smoking. A Cochrane review discussed the lack of trials evaluating smoking cessation and the evolution of disease in patients [48].

There are approximately 20 substances in cigarette smoke that produce carcinogenic effects. The most important of these substances are nitrosamines, polycyclic aromatic hydrocarbons, aromatic amines, and aldehydes. Nicotine in tobacco has no

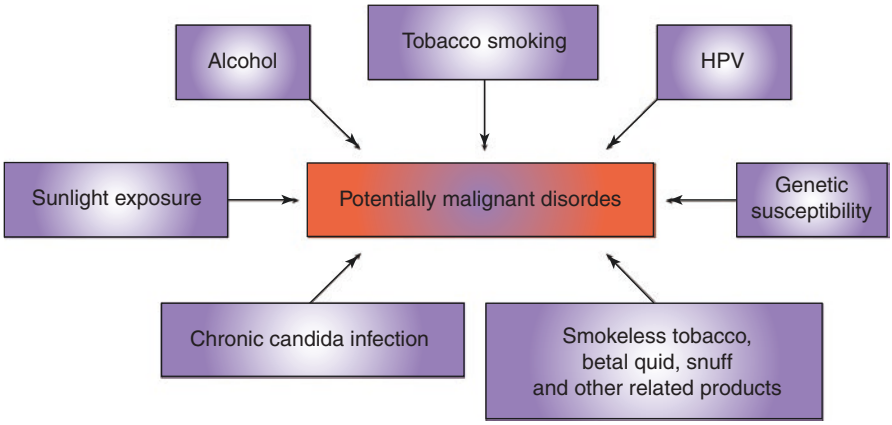

Fig. 2.3 Aetiological factors of oral potentially malignant disorders

carcinogenic effect, but it is a highly addictive substance. In general, tobacco products are carcinogenic only after metabolic activation; however, host enzymes can detoxify them.

Nitrosamines are found in smoked and smokeless tobacco, and their metabolites can covalently bind to DNA, forming DNA adducts that can promote mutations [49]. However, the carcinogenic effect of nitrosamines requires metabolic activation. In addition to forming DNA adducts, nitrosamines generate hydroxyl radicals or other reactive oxygen species that can damage DNA and cause single-strand breaks. Benzopyrene, a polycyclic aromatic product, and aromatic amines can cause mutations in *TP53* and the formation of different DNA adducts. Acrolein, an aldehyde present in tobacco, is an active carcinogenic product and is associated with mutations in *TP53*. Acrolein adducts inhibit nucleotide excision repair enzymes, which, as discussed earlier in this chapter, is an important mechanism for the repair of DNA damage caused by tobacco products.

It is interesting that tissues directly exposed to tobacco products, as well as those not directly in contact with them, show elevated levels of DNA adducts in smokers. A recent study demonstrated a predominance of T>C and C>T mutations in oral cancer cells in smokers, and these alterations were correlated with age at the time of diagnosis of the disease [50].

Tobacco products may also cause methylation of tumour suppressor genes, induction of oxidative stress, and inflammatory reactions. Oral cancer cells in smokers contain more hypomethylated and hypermethylated genes than non-smokers, indicating a change in the normal methylation pattern. Recent studies have also demonstrated altered expression of miRNAs in tobacco-related neoplasias.

Smokeless Tobacco, Betel Quid, Snuff, and Other Related Products

Smokeless tobacco is a term used to define the consumption of tobacco without burning, and it is a risk factor for OPMD. There are a variety of smokeless tobacco products that can be chewed, sucked on, or sniffed. They also can be used together with other ingredients such as areca nut, lime, spices, and ash. Tobacco is sometimes boiled or burned for consumption. Smokeless tobacco can cause the formation of DNA adducts and the production of reactive oxygen species, which can cause mutations in several genes, including *HRAS*, *KRAS*, *NRAS*, and *TP53* [51]. Smokeless tobacco also may cause disruption of the cell cycle by the hypermethylation of tumour suppressor genes [52].

Betel-related products for chewing or betel quid usually include betel leaf, lime, tobacco, and betel nuts. Betel quid has two basic carcinogenic actions in the oral mucosa. The first is the cytotoxic and mutagenic effect of its components (arecoline, alkaloids and polyphenols) on epithelial cells, while the second is associated with induced fibrosis, which reduces the oxygen supply to the epithelial cells.

Chewing betel quid is strongly associated with the development of oral submucous fibrosis, which is an important OPMD that occurs specially in South Asia [53]. The mechanism by which betel quid produces submucous fibrosis in oral tissues involves the action of its different components. This mechanism mainly involves suppression of endothelial cell proliferation; generation of reactive oxygen species; activation of NF-kB, JNK, and p38 pathways; production of connective tissue growth factors; and upregulation of TGF-b. These alterations cause DNA damage, progressive accumulation of collagen, and cross-linking of collagen fibres, which renders them less susceptible to breakdown. These effects explain the fibrotic nature of the disease, and the loss of vascularity leads to atrophy of the epithelium.

Recent studies have also suggested that areca nut compounds are involved in the epithelial-mesenchymal transition [54]. The epithelium-mesenchymal transition phenomenon has an important role in differentiation, migration, and invasion of keratinocytes, and it has been implicated in the malignant transformation of oral submucous fibrosis. Other molecular changes induced by betel components include the overexpression of CAIX, a hypoxia-inducible enzyme overexpressed by cancer cells, and the decreased expression of tumour suppressor genes, such as *PTEN* and *BRCA* protein-related genes.

Alcohol

The role of alcohol in OSCC is more clearly established than in the development of oral leukoplakia. A prospective study reported by Maserejian et al. (2006) [55] demonstrated that alcohol consumption is an independent risk factor for oral leukoplakia; however, this finding was not confirmed definitively by other reports. The independent risk effect of low/moderated alcohol consumption is unclear, considering the different types of beverages available.

Alcohol dehydrogenase (ADH) catalyses the oxidation of alcohol to acetaldehyde, which is the major metabolite of alcohol [56]. This process occurs in the cytoplasm. In chronic alcohol consumption, the CYP2E1 enzyme is utilized and results in acetaldehyde formation in peroxisomes. Acetaldehydes are very toxic and affect DNA synthesis and repair. Because of its electrophilic nature, acetaldehyde can bind and form adducts with proteins, lipids, and DNA, which impairs their functions and promotes DNA damage and mutation. The carcinogenic effect of alcohol is also mediated by increased oxidative stress, release of inflammatory cytokines, impairment of retinoid metabolism, and inhibition of DNA methylation.

As acetaldehyde is toxic and can cause health problems, it needs to be oxidized to acetate by the enzyme aldehyde dehydrogenase [56]. As the acetate formed is unstable, it breaks down spontaneously to CO_2 and water. Genetic factors can influence the propensity for the accumulation of acetaldehyde. SNPs in the alcohol

dehydrogenase and aldehyde dehydrogenase genes can result in the toxic accumulation of acetaldehyde, thereby enhancing its procarcinogenic effect.

HPVs

Human papillomaviruses (HPVs) are small double-stranded DNA viruses, and their family consists of more than 130 types, including high-risk and low-risk types [57]. Among the many high-risk HPVs, HPV-16 is the most common, and it accounts for approximately 90% of HPV-positive carcinomas of the oropharynx [58]. These viruses are sexually transmitted primarily through direct contact, and the majority of infections clear spontaneously within 24 h; however, this does not necessarily create immunity. HPV-positive head and neck cancers, when compared to HPV-negative counterparts, affect younger patients and are less likely to be associated with risk factors such as smoking and alcohol [59]. While less than 5% of non-oropharyngeal head and neck cancers are caused by HPV infection, greater than 70% of oropharyngeal cancers are related to this virus [60, 61]. A recent meta-analysis suggested that HPV16 is a significant independent risk factor for oral leukoplakia [58].

Recent studies have demonstrated molecular mechanisms in which HPVs induce carcinogenesis. E6 and E7 HPV proteins function as the dominant oncoproteins of high-risk HPVs, and they inactivate the tumour suppressor proteins p53 and pRB, respectively [57]. *TP53* is the "guardian of the genome", and its malfunction in most cancers is the result of DNA mutation. In HPV-associated cancers, the E6 oncoprotein degrades the wild-type p53 protein and leads to chromosomal instability in a manner similar to of DNA mutations. HPV E7 protein inactivates pRB, which releases E2F and promotes the transition from the G1 to the S phase of the cell cycle by transcription of the cyclins E and A. The disruption of pRB causes overexpression of p16, which explains why the overexpression of p16 is one of the markers of the infection used in immunohistochemistry. The immunohistochemical study of p16 protein in conjunction with in situ *hybridization* is the gold standard for the diagnosis of HPV-associated cancer. HPV-negative oropharyngeal cancer is associated with approximately twofold more mutations than the HPV-associated counterpart. HPV-positive head and neck cancer has an improved prognosis; however, its precursor lesion in the oropharynx has not yet been identified.

Chronic Candida Infection

Despite the extent of the oral presence of *Candida albicans* being higher in patients with OSCC or oral leukoplakia, the role of this microorganism in oral carcinogenesis is not well established [62, 63]. *C. albicans* produces nitrosamines that are

important carcinogenic compounds. Nitrosamines, after metabolic activation by cytochrome P450 enzymes, induce alkylating DNA damage by formation of the highly reactive diazonium ion, which leads to mutations in DNA. Point mutations can activate specific oncogenes or suppress tumour suppressor genes, as discussed earlier in this chapter. Additional potential mechanisms by which *Candida* spp. may promote oral carcinogenesis include the inflammatory reaction associated with infection and the metabolism of ethanol with the consequent production of acetaldehyde, a potential carcinogenic compound.

Sunlight Exposure

Long-term exposure to sunlight is the major aetiological factor of cancer in the lower lip. Actinic cheilitis is an OPMD of the lower lip, and it can progress to squamous cell carcinoma. There are three types of ultraviolet radiation (UV) that can damage the genome: UVA (315–400 nm), UVB (280–315 nm), and UVC (100–280 nm). UVB and UVC can produce DNA photoproducts, including pyrimidine photoproducts. These photo lesions can cause UV signature mutations (C>T transitions and CC>TT tandem double mutations), leading to upregulation and downregulation of signal transduction pathways and cell cycle dysregulation [64]. The CC>TT transition in *TP53* has been reported in lip squamous cell carcinomas as well as in actinic cheilitis. Additional effects of UV include the depletion of antioxidant defences and the induction of local immunosuppression. Nucleotide excision repair enzymes are able to repair DNA by removing UV-induced photo lesions [65]. Therefore, nucleotide excision repair enzymes counteract the formation of mutations and the development of skin/lower lip cancers.

Genetic Susceptibility to OPMD

Although the risks of lifestyle exposures to environmental carcinogens are associated with the development of premalignant lesions in the oral mucosa, genetic susceptibility helps to explain interindividual or interpopulation variations. Most studies are dedicated to the investigation of genetic risk factors for the development of oral cancers, and few of them are focused on OPMD.

A variation in a single nucleotide that occurs at a specific position in the genome is known as a single nucleotide polymorphism (SNP) (reviewed earlier in this chapter), and it can change the amino acid sequence of a protein. This change can affect the protein's function and its ability to metabolize carcinogens or its capacity to repair DNA damage caused by a carcinogenic substance.

Carcinogenic compounds related to oral cancer can be activated or degraded by a certain group of enzymes known as xenobiotic metabolizing enzymes (XMEs).

The metabolism of tobacco products, for example, involves oxygenation by P450 enzymes in cytochromes and conjugation by glutathione-*S*-transferase. Many XME SNPs can influence the individual's biological response to carcinogens. Because of the mutagenic effect of acetaldehyde, SNPs in the enzymes involved in alcohol metabolism (alcohol dehydrogenase and aldehyde dehydrogenase) are also related to the risk of developing oral cancer [66].

Genotype variations associated with increased susceptibility to the development of OSCC also include genes related to inflammation, stabilization of the genome, regulation of cell proliferation, apoptosis, and tumour survival [66]. Therefore, SNPs can partly explain the genetic susceptibility to human diseases, including the development of oral cancers and potentially malignant lesions. The investigation of SNPs may be helpful in identifying patients who are affected by OPMDs that may present an increased risk for malignant transformation.

Conclusion

Histopathological examination is not sufficient to accurately predict the malignant potential of OPMD. Despite being the gold standard method for assessing the grade of dysplasia, mild dysplastic lesions may progress to OSCC, while lesions with higher dysplastic features may not suffer malignant changes. For example, only approximately 5% of oral leukoplakias progress and transform into OSCC. However, the challenge is to identify which lesions are at risk and which lesions will never progress. Therefore, several studies have attempted to identify molecular changes associated with the malignant progression of oral leukoplakia.

As cancer development and progression indicate instability in the genome, this feature has been studied in OPMD, and chromosomal instability was reported to be a reliable method for the assessment of premalignant lesions of the oral mucosa at risk for transforming into cancer [67]. Other malignant transformation markers are beginning to be identified. LOH patterns were shown to be able to predict oral leukoplakia lesions at risk for malignant transformation. Epigenetic changes are also relevant to malignant progression, and hypermethylation of p16 is apparently associated with a higher potential of oral leukoplakia malignant transformation [68]. Specific miRNAs were demonstrated to be overexpressed in oral leukoplakia that progressed to oral cancer, and some cytological and histopathological parameters used to grade dysplasia are associated with altered expression of miRNA [13, 14].

There are several layers of complexity that surround the oral malignant transformation issue. One needs to keep in mind that the individual interacts with the environment (and potential carcinogen sources) as the epithelium interacts with the microenvironment (extracellular matrix, blood vessels, fibroblasts, immune cells, etc.) and the genome interacts with the epigenome. In addition, the utility of molecular and histopathological profiling is limited by intra-lesional heterogeneity, which may in part explain the discordant results in the literature.

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