Understanding the complex pathogenesis of oral cancer: A comprehensive review

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The pathogenesis of oral cancer is a complex and multifactorial process that requires a deep understanding of the underlying mechanisms involved in the development and progress of malignancy. The ever-improving comprehension of the diverse molecular characteristics of cancer, the genetic and epigenetic alterations of tumor cells, and the complex signaling pathways that are activated and frequently cross talk open up promising horizons for the discovery and application of diagnostic molecular markers and set the basis for an era of individualized management of the molecular defects underlying and governing oral premalignancy and cancer. The purpose of this article is to review the key molecular concepts that are implicated in oral carcinogenesis, especially focusing on oral squamous cell carcinoma, and to review selected biomarkers that play a substantial role in controlling the so-called "hallmarks of cancer," with special reference to recent advances that shed light on their deregulation during the different steps of oral cancer development and progression. (Oral Surg Oral Med Oral Pathol Oral Radiol 2021;132:566−579)

Similar to any other disease, proper diagnosis and management of oral cancer requires a thorough understanding of the underlying mechanisms that are involved in its development and progression. However, the pathogenesis of oral cancer (or oral carcinogenesis) is still considered a quite complicated process, the limited comprehension of which contributes to persistently high incidence and poor outcome.[1] In recent years, significant advances have been made in the field of molecular biology and numerous studies have elucidated the basic processes that play a central role in carcinogenesis of several types of malignancy, including oral cancer. Ideally, these processes can be manipulated through strategically planned interventions to diminish oral cancer incidence (through early detection and eradication at a premalignant state) and mortality (through individualized targeted management).

Indeed, a better understanding of the development and progression of malignant neoplasms and their underlying molecular events has allowed the gradual implementation of clinical applications, ameliorating prevention and management of several types of cancer. It is now widely accepted that the diversity of cancer not only applies to the clinical and pathologic findings but is also associated with, and actually driven by, an array of variegated genetic and epigenetic alterations.[2,3] Special emphasis has also been given to the immunologic response during development of different types of cancer that may exhibit heterogeneity in their immune landscape with distinct immune signatures.[4] All of these molecular events exhibit significant variations not only among different types of malignant neoplasms (e.g., breast cancer, melanoma, lymphoma, etc.)[2] but also among distinct subtypes of the same type of cancer (e.g., human papilloma virus−positive and −negative [HPV+ and HPV−] oropharyngeal cancer).[5] Taking advantage of the sophisticated new technologies in the field of molecular biology, it is now plausible to set the goal of identifying a distinct genetic profile in each neoplasm, allowing an individualized treatment that targets the specific molecular defects of each case.[6]

The aforementioned notions can be applied in oral cancer and in head and neck cancer in general, the most common malignant neoplasms worldwide.[1] The recent major discoveries in head and neck cancer research create new opportunities as well as demands for the future. The purpose of this article is to review the key molecular concepts that are implicated during oral carcinogenesis.

Statement of Clinical Relevance

A comprehensive understanding of the molecular basis of oral carcinogenesis is a prerequisite for critical evaluation of the current oral premalignancy and cancer literature and constitutes fundamental knowledge for identification of molecular biomarkers and targets allowing individualized diagnosis and treatment.

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Considering that squamous cell carcinoma (SCC) constitutes the vast majority of oral cancer cases and is by far the most studied and better characterized form of oral malignancy, the present review focused on oral SCC (OSCC) carcinogenesis and selectively reviewed the vast body of relevant literature. Additionally, biomarkers related to the previously described "hallmarks of cancer"[3,7] are presented with specific reference to their deregulation during different steps of oral cancer development and progression.

BASIC CONCEPTS IN ORAL CARCINOGENESIS

Multistep oral carcinogenesis

Similar to other types of malignant neoplasms, oral cancer (and, more specifically, OSCC) occurs as a result of a multistep process that is characterized by various distinct genetic and epigenetic alterations.[8,9] Independent of various exogenous (e.g., smoking, alcohol use, oncogenic HPV types) or endogenous factors (e.g., genetic predisposition and rare syndromes; e.g., Fanconi anemia) implicated in cancer,[10] a fundamentally common feature in oral carcinogenesis is the gradual accumulation of molecular defects, including mutations, chromosomal abnormalities, epigenetic alterations, and others.[8,11] All of these changes collectively initiate phenotypic (clinical and microscopic) transformation from normal epithelium to dysplastic and finally to invasive carcinoma (Figure 1).[12]

Although the aforementioned stepwise transition from normal epithelium to invasive cancer through a progressively worsening premalignant state (at the molecular, microscopic, and clinical levels) generally holds true, this should not be construed as a straightforward succession of events. Specifically, at the microscopic level, it is not necessary to go through all grades of dysplasia (i.e., from mild to moderate to severe to carcinoma in situ) before development of cancer cell invasion of and through the basement membrane zone; it is possible to encounter invasive cancer cells in the underlying connective tissue without severe or full-thickness dysplastic changes in the overlying epithelium.[13,14] Indeed, acquisition of invasive properties by epithelial cells is a complex process that involves their interaction with connective tissue elements and the immune system[15,16] (as described later), and it is not solely dependent on a simplistic linear or quantitative expansion of dysplastic features.

Accordingly, at the molecular level, the progressive accumulation of molecular changes that eventually culminates in the development of invasive cancer is by no means a straightforward succession of alterations; there is not a certain number, combination, or particular order or timeline of events that needs to happen before malignancy occurs. Instead, different combinations of specific molecular changes, affecting a multitude of biological processes, may result in the same fundamental phenotypic change of oral cancer; that is, invasive growth.[12] It is also possible that the genetic alterations that are necessary for the initial steps of carcinogenesis, which phenotypically correspond to the transformation of normal epithelial cells into dysplastic ones, are higher in number compared with the additional aberrations that lead to transition from dysplasia to malignancy.[12] Recent studies have also supported the notion that the initial steps of premalignancy (e.g., oral leukoplakia without dysplasia) are molecularly discrete compared with dysplastic lesions.[17] On the other hand, other investigators have proposed that dysplastic and

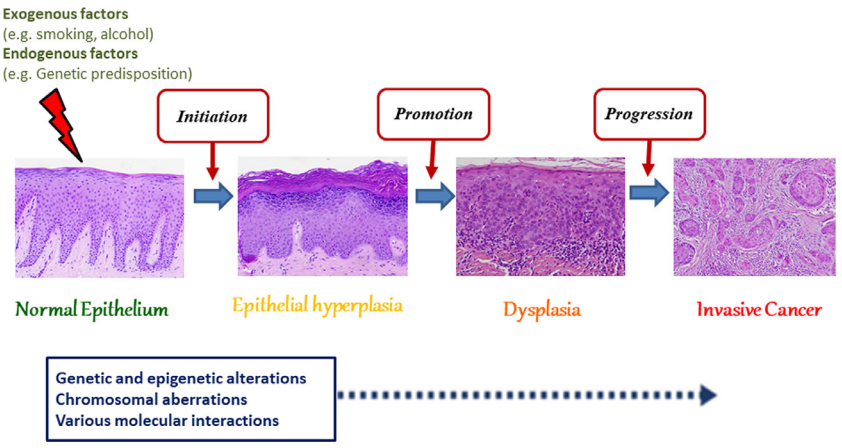

Fig. 1. Simplified scheme of the progressive nature of oral carcinogenesis. Under the influence of various exogenous or endogenous factors, the normal oral epithelium undergoes molecular changes that accumulate and over time lead to malignant transformation. The transition from normal epithelium to the various precancerous stages and finally to invasive cancer is characterized by the cumulative acquisition of various molecular characteristics at various time points (traditionally divided into phases of initiation, promotion, and progression). This process, which frequently occurs over an extended period, involves clonal selection and evolution favoring the development and progression of malignancy.

nondysplastic oral leukoplakias, both capable of transforming into malignancy, demonstrate similar molecular alterations.[18] Furthermore, even after cancer development, subsequent genetic alterations take place and increase the potential of cancer cells to invade, metastasize, and evade elimination by the immune system.[11,19] To date, several studies characterizing the underlying molecular events that are associated with malignant transformation of precancerous lesions have been reported; however, further longitudinal experimental studies need to be performed in order to establish clinically useful prognostic biomarkers in oral potentially malignant disorders.[12,20]

Tumor evolution, monoclonality, and stem cells

Monoclonality is a basic perception in carcinogenesis and refers to the derivation of all cancer cells from a common progenitor that underwent the initial genetic defects.[21,22] Tumor evolution (TE) is the process of development of a tumor mass initiated by alterations of a single cell in the normal tissue.[22] The survival of precursor cells and of their derivatives, which also requires resistance to repair mechanisms, apoptosis, and immune surveillance, gives genesis to specific clones that gradually increase in size, eventually resulting in establishment of malignancy.[3] Intratumor heterogeneity (ITH) is the result of the formation of genetically distinct subclones within the tumor mass.[23] Even though new mutations take place in different cells of a common clone during the progression of malignancy, causing gradually increasing variation from their precursors and molecular diversity between the cancer cell population, all cells of a given cancer share genetic features that support their common origin.[21]

There are 4 main different TE theories in the literature: linear, branching, neutral, and punctuated.[22] Linear evolution, the most well-known concept of TE, supports that driver mutations (those that confer a fitness advantage) are gradually acquired by the "fittest" or dominant clone that finally outcompetes the others; this stepwise process leads to progressively greater stages of malignancy through successive selective sweeps.[24] Branching evolution theory hypothesizes that a common ancestor gives rise to different clones that coexist and develop in parallel within the tumor mass, all characterized by increased fitness, with infrequent selective sweeps.[25] Neutral evolution theory, a variation and extreme case of the branching model, suggests that numerous clones acquire random mutations without conferring any particular fitness advantage, thus causing enormous ITH.[26] Finally, the punctuated (also known as "big bang") concept of TE claims that all mutations occur in short periods of time at the earliest cancer stages,[27] overriding the aforementioned theories that endorse sequential acquirement of genetic aberrations; instead, high ITH is expected at the beginning of tumor development with subsequent expansion of one or a few dominant clones.

Few studies investigating TE models, as well as the prognostic value of ITH in oral and head and neck SCC, have been reported in the literature. A study of patients with distant metastasis of primary head and neck cancer using massively parallel sequencing revealed that a branched pattern of evolution was evident in 31.9% of cases and was associated with metastasis in distant anatomic regions in a shorter period of time and with a trend to worse overall survival; however, in the majority of patients, a more stable model resembling punctuated evolution was detected (68.1%).[28] The clinical significance of ITH in head and neck SCC was also supported by a molecular analysis using next-generation sequencing (NGS) in 74 patients: A strong association between high MATH (mutant-allele tumor heterogeneity, a unique quantitative measurement of ITH based on NGS) levels and poor clinical outcome was revealed.[29] A subsequent multi-institutional study by the same group using samples of 305 patients with head and neck SCC further established the significant negative correlation between high MATH and overall survival.[30] More specifically for oral cancer, high levels of ITH, as well as intrafield heterogeneity (IFH), have been detected by applying NGS techniques in SCC and adjacent mucosa specimens[31]; in a subsequent study, the same group compared ITH and IFH between 5 recurrent and 5 nonrecurrent OSCC specimens and concluded that ITH was frequent in both recurrent and nonrecurrent groups, and high IFH was correlated with higher risk of developing locoregional recurrences and/or second primaries.[32] Whole exome sequencing analysis revealed a closer genomic profile in 2 well-differentiated oral SCC samples compared with 2 poorly differentiated cases and an inverse correlation between heterogeneity and physical separation (distance between samples) was noticed. However, the authors suggested that the pattern of TE could be either linear or not linear.[33] Finally, by performing whole-genome sequencing (WGS) on separate regions of an HPV-related oropharyngeal SCC case, Zhang et al.[34] demonstrated the presence of widespread ITH within the primary and the metastatic sites, also suggesting a branching evolution process.

In recent years, specific emphasis has been given to the role of stem cells in cancer.[35] Regarding oral carcinogenesis, it has been proposed that a stem cell of the basal cell layer of the squamous epithelium could be a progenitor of a cancer clone.[36] These stem cells possess properties that facilitate the acquisition of neoplastic features that give rise to a specific malignant clone.[36] It has also been suggested that among all cancer cells, only a small subset continues to display features

consistent with stem cells.[37] This subpopulation of cancer cells has been implicated in the development of metastasis, multiple recurrences, and resistance to chemotherapy and, as a result, has been a target of contemporary treatment modalities.[35-38] Conventional cancer stem cell markers that have shown deregulated expression during the different steps of oral carcinogenesis and may be associated with progression of malignancy in oral potentially malignant disorders, as well as poor prognosis and resistance to chemo- and radiotherapy in oral cancer, include OCT4, SOX2, CD44, CD24, CD133, and ALDH1, among others.[39]

Field cancerization

After the development of the initial clone, new mutations provide the ground for further survival and expansion of the clonal neoplastic population.[40] Clonal cells proliferate aberrantly as a manifestation of their ability to evade DNA repair mechanisms, apoptotic signals, and immune recognition.[11] Gradually, the cells of a premalignant clone undergo intraepithelial expansion, not only upwards throughout the epithelial layers but also laterally along the peripheral margins. This lateral expansion, during which the adjacent normal keratinocytes are replaced by genotypically clonal cells (with or without phenotypic dysplastic alterations), leads to the development of an extended "field" in a process called "field cancerization."[21] Further to the possibility of lateral propagation, field cancerization has also been etiologically attributed to exposure of all oral mucosa surface (as well as of adjacent anatomic areas, such as pharynx and larynx) to carcinogenic factors (primarily smoking and alcohol use), resulting in accumulation of defects, not necessarily of common origin, over a wide area (or several independent fields) of the surface epithelium.[21] Regardless of the underlying initiating mechanisms, the clinical significance of field cancerization cannot be underestimated. There is no doubt that the created field could extend to a surface diameter of several centimeters, extending far beyond the clinical and microscopic limits of the observed lesions. This explains the difficulty of effectively treating potentially malignant and malignant disease, even in cases of radical surgical management, and sets the ground for development of diffuse and/or multifocal premalignant lesions as well as an increased number of recurrences or second primary cancers.[11,21,40]

Oncogenes and tumor suppressor genes

From a molecular standpoint, cancer development relies on the activation of signals and processes with tumor promoting effects and the inhibition of those with tumor suppressive properties. The genes that induce the aforementioned changes by encoding proteins that promote or inhibit malignant transformation and progression are called oncogenes and tumor suppressor genes, respectively.[41] New biomarkers are constantly being discovered and the molecular pathways that are implicated in carcinogenesis are much better characterized over time. These molecular defects deregulate the basic cellular processes, including cell cycle, proliferation, differentiation, metabolism, senescence, and apoptosis, and evoke changes in the interaction of cancer cells with the tumor environment, affecting angiogenesis, inflammation, and immune mechanisms and facilitating the development and expansion of the neoplastic clones.[3,11]

The biologic mechanisms of activation of oncogenes and inhibition of tumor suppressor genes are variable and complicated (e.g., chromosomal aberrations, mutations, epigenetic modifications). Specifically in oral carcinogenesis, special attention has been given to loss of heterozygosity and aneuploidy, as well as other epigenetic modifications and defects in signaling pathways,[12,42] as briefly presented in the following subsections.

Loss of heterozygosity and aneuploidy

A large variety of chromosomal abnormalities have been detected during the different steps of oral carcinogenesis, using several molecular tools, including karyotype analysis, fluorescence in situ hybridization, and polymerase chain reaction. Loss of heterozygosity (LOH) and microsatellite instability have been recorded in high frequencies among potentially malignant and malignant lesions of the oral mucosa.[43] More specifically, dysplastic lesions have been associated with defects in chromosomal regions 9p21, 3p14, and 17p13, which harbor genes with substantial roles in cellular functions implicated in malignant transformation: the *P16^{INK4A}/CDKN2A* gene is located in 9p21, the *FHIT* gene in 3p14, and the *TP53* gene in 17p region.[42-44] Additionally, the probability of malignant transformation of precancerous lesions seems to be related to LOH at specific chromosomal regions.[45,46] Further accumulation of chromosomal defects is connected with transition to carcinoma in situ (in which LOH in 11q13, 13q21, and 14q31 is observed) and invasive carcinoma (LOH in 4q, 6p, 8p, and 8q).[43]

Additionally, cancer cells are usually characterized by aneuploidy, which represents anomalies in the number of chromosomes as a result of chromosomal instability. A high percentage of oral dysplastic lesions and malignancies exhibit aneuploidy. Interestingly, the degree of dysplasia as well as the risk of malignant transformation in oral potentially malignant disorders is positively related with the presence of aneuploidy.[47-49] It is also acknowledged that aneuploidy develops during the initial steps of carcinogenesis and tends to accumulate and become more frequent as the malignancy progresses.[50]

Epigenetic alterations

Apart from mutations that affect the sequence of DNA, regulation of gene expression is also a result of epigenetic mechanisms, including DNA methylation, histone modifications, and posttranscriptional silencing.[8,51] These epigenetic modifications, especially methylation of gene promoter regions, are also considered significant methods of regulating expression of genes that are implicated in oral carcinogenesis.[8] Methylation usually takes place in regions with increased G-C content and, as a result, prevents the binding of a transcription factor to the DNA.[51] Increased gene methylation has been detected in phenotypically normal oral mucosa adjacent to cancer tissue, leading to the conclusion that DNA methylation events occur early in oncogenesis. Accordingly, hypermethylated genes have been observed at increased percentages in dysplastic lesions of the oral cavity, including CDKN2A/p16INK4a, p14ARK, p15, p53, CDH1, MGMT, DAPK1, GSTP1, and RARB.[2,8,42] Posttranslational modifications affecting histones (e.g., methylation or acetylation of lysine residues in histone tails, presence of the γ-H2A.X histone variant, or deregulated expression of histone deacetylases) may affect the packaging of chromatin, thus affecting the expression of genes that are implicated in oral cancer.[8,52]

In addition to methylation of promoter regions and histone modifications, which are considered classic and well-established epigenetic events that affect gene expression, other RNA-mediated epigenetic alterations have gained prominence in recent years.[8,53] It has become apparent that not all RNA molecules encode for proteins, and a wide spectrum of ribonucleic acids that do not undergo translation (so called noncoding RNAs, ncRNAs) display distinct functions, some of which include epigenetic regulation of gene expression.[54] Recent studies have emphasized their decisive role in various normal biological functions and disease progression, including cancer.[53,55] These include microRNAs (miRNAs or miRs) and the newly emerging long ncRNAs (lncRNAs) and circular RNAs (circRNAs). miRNAs are single stranded RNAs, 19 to 25 nucleotides long, that most commonly interact based on complementarity of their seeding sequence with a 3′ untranslated region of an mRNA, inducing degradation of the later (posttranscriptional regulation of gene expression) or by inhibiting translation at the postinitiation step.[56] The role of miRNAs in carcinogenesis by regulating gene expression of oncogenes as well as tumor suppressor genes has been emphasized.[57] Similar to the functional classification of genes, miRNAs can be classified as those promoting or suppressing onocogenesis (onco-miRs and onco-suppressive-miRs, respectively). They have been implicated in various functions, including cell proliferation, apoptosis, differentiation, and others, and they affect the ability of cancer cells to invade and metastasize.[58] Regarding oral premalignant lesions and cancer, differences in the expression levels of a significant number of miRNAs (including miR-21, miR-31, miR-345, and others) have been established in tissues, blood, or saliva samples, rendering these molecules as potential diagnostic and prognostic biomarkers and promising therapeutic targets.[42,58,59] lncRNAs are distinct molecules exhibiting specific structural characteristics (>200 nucleotides long) and, in contrast to miRNAs that mainly interact with mRNA (and less commonly with the promoter regions of genes), they can interact with either DNA, RNA, or a protein.[60] This results in a diversity of functions to the extent that they have been implicated in almost every step of gene expression from transcription regulation (through chromatin remodeling as well as through scaffolding of transcription factors to the promoter region of a gene) to mRNA turnover (e.g., through interactions with miRNAs or through direct association with mRNA that causes decay of the later), translational initiation (e.g., inhibition of translation), and protein-protein interactions (protein scaffolding).[60] Hence, it is obvious that their deregulation could be a significant phenomenon associated with cancer development and progression.[61] In terms of oral cancer, a large number of lncRNAs exhibit abnormal expression levels in OSCC tissues, showing either increased expression (onco-lncRNAs), including MALAT1, CCAT1, UCA1, and HOTAIR, or decreased expression (tumor suppressor lncRNAs), including MEG3.[62,63] Additionally, detectable levels of HOTAIR and MALAT1 in the saliva of patients with OSCC have been reported.[64] The detection of the former in the saliva of patients with metastatic disease also highlights the potential role of salivary HOTAIR in predicting metastasis in OSCC.[65] Finally, recent transcriptomic analyses in dysplastic lesions of the oral mucosa rendered a large amount of lncRNAs that are differentially expressed (such as NEAT1); however, new studies need to be conducted to better characterize the functional effect of lncRNA regulation of gene expression in oral premalignancy.[65] circRNAs are recently characterized circular single-stranded RNA molecules that are generated as a result of back-splicing of a precursor mRNA.[66] Interestingly, even though circRNAs are included in the category of noncoding RNAs, they can also be translated into proteins.[67] Other functions include acting as sponges for miRNA or interacting with RNA-binding proteins, mRNAs, or the RNA polymerase II machinery, causing control of gene expression at various levels.[66,68] In addition to their role in physiologic functions, circRNAs are involved in the pathogenesis of several diseases, including cancer, by affecting the expression of

oncogenes or tumor suppressor genes.[68,69] Regarding oral cancer, recent findings support the differential expression of many circRNAs, some of which have also been shown to be deregulated in other types of malignancy.[70] Certain circRNAs showed increased expression in OSCC samples compared with control tissues (onco-circRNAs, including Circ_0001821 and Circ_0002185), and others were downregulated in oral cancer (tumor suppressor circRNAs, such as Circ_0086414, Circ_0072387, Circ_0008309, Circ_001242, and Circ_0092125). The latter molecule also been associated with prognostic parameters (low expression correlated with higher TNM stage, larger tumor size, increased metastatic potential, and decreased survival).[71] A recent study showed that circANTRL1 may be associated with enhanced radiosensitivity of oral cancer through inhibition of miR-23a-3p resulting in upregulated expression of PTEN, highlighting the potential of this molecule as a therapeutic target for better response to radiotherapy.[72] Finally, emerging studies in the field of oral premalignancy detected a large panel of circRNAs that are differentially expressed in oral leukoplakia (among which circHLA-C showed the most significant upregulation and also correlated with the degree of dysplasia).[73]

Molecular signaling pathways

Various signaling pathways have been proven to be deregulated during carcinogenesis of the oral mucosa, exhibiting complex interactions. In many instances, these deregulations result in overactivation of oncogenic signaling pathways as a result of overexpression or mutation of their receptors, ligands, or downstream molecules.[74] Although a complete list of deregulated signaling pathways and their cross talk is beyond the scope of this article, some of the main signaling pathways in the context of oral carcinogenesis include the epidermal growth factor receptor (EGFR) pathway, the Ras-Raf-mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway, as well as the Janus-kinase/signal transducer and activator of transcription (JAK/STAT) pathway.[9,11,12]

Among the most significant pathways controlling cellular proliferation and apoptosis of oral epithelial cells, particular attention should be paid to those mediated by receptor tyrosine kinases including EGFR (ErbB1).[75,76] Overexpression of EGFR has been observed in oral premalignancy and cancer and has been associated with the recruitment and activation of various oncogenic molecules, including RAS, PI3K, and Stat3 (through the MAPK, PI3K/Akt, and JAK/Stat3 pathways, respectively), resulting in a cascade of downstream activation of molecules regulating differentiation, proliferation, apoptosis, angiogenesis, and metastasis.[75,77] More precisely, the Ras/Raf/MEK/ERK signaling cascade is activated by the excessive activity of EGFR (via overexpression of the receptor and/or its ligands like EGF or transforming growth factor [TGF]-α) or other growth factor receptors, resulting in activation of transcription factors that target the expression of specific genes (like c-fos). It is also very commonly involved in carcinogenesis of the oral mucosa and has been explored as a potential therapeutic target.[78,79] PI3K/Akt has also been a subject of extensive studies and has been shown to be frequently mutated in OSCC.[80] Additionally, disturbances of this pathway have been noted in oral potentially malignant disorders and have been associated with increased risk for malignant transformation.[81] PI3K binds to phosphorylated receptor tyrosine kinase and generates a series of events that activate Akt, which affects various downstream molecules including mTORC1, MDM2, pro-caspase 9, and bcl-2.[82] MDM2 inhibits p53, and this event, in addition to the inactivation of pro-caspase 9 and activation of bcl2, inhibits cell cycle arrest and apoptosis. On the other hand, mammalian target of rapamycin (mTOR) phosphorylates the fundamental transcription factors S6K and 4EBP1.[11,82] It is also noteworthy that the aforementioned pathways exhibit a significant cross talk, mainly through positive regulation (e.g., activation of mTORC1 by ERK), which highlights the idea that once the upstream event takes place (tyrosine kinase receptor activation), downstream synergistic cascades generate tumor promoting functions.[80]

Another signaling pathway playing a key role in the pathogenesis of oral cancer is the JAK/Stat3 pathway, which is commonly overexpressed even from the initial stages of oncogenesis.[83] Stat3 is activated either through EGFR or through JAK/cytokine receptor signaling and acts as a transcription factor for genes that regulate cellular proliferation and apoptosis, such as cyclins, myc oncogene, survivin, and the bcl family.[8,84] The Stat3 pathway also interacts with other important pathways, such as MAPK and nuclear factor κ B (NF-κB) via IL-6.[84-86]

HPV-related molecular carcinogenesis

It is well established that a subset of SCCs of the oral cavity and much more frequently of the oropharynx are etiologically associated with high-risk (HR) oncogenic HPVs, exhibiting specific biologic, histopathologic, and prognostic differences compared with non-HPV–related carcinomas.[87,88] Additionally, in some cases oral premalignancy may exhibit association with HPVs, showing unique microscopic characteristics and possibly distinct biological behavior.[89] Hence, an understanding of the main underlying molecular mechanisms in HPV-related oral carcinogenesis is needed.

These mechanisms are similar to those that take place in cervical cancer development and mainly revolve around the action of 2 viral oncoproteins: E6 and E7.[90,91] A requirement for HR HPV−induced malignant transformation is the incorporation of the viral genome into the host DNA in the epithelial cells of the basal layer, which permits the aforementioned viral oncoproteins to exert their profound effects on key points of the cell cycle.[90] Precisely, the E6 oncoprotein of HR-HPVs targets the p53 tumor suppressor protein, causing its proteolysis via ubiquitination. In turn, p53 degradation results into deregulation of DNA repair mechanisms and inhibition of apoptosis, promoting oncogenesis.[88,91-93] On the other hand, E7 interacts and silences the tumor suppressor gene of retinoblastoma (Rb), which under normal conditions regulates the cellular proliferation by its protein product retinoblastoma protein (pRb).[42,88,91] This silencing leads to aberrant cellular divisions and allows the cell to enter a state of senescence, which promotes carcinogenesis. It is also significant that decreased activity of pRb results in overexpression of p16, which is widely used as a surrogate marker for HPV infection in SCC of the head and neck with a higher reliability in oropharyngeal cancer compared with oral cancer.[11,88,94]

THE HALLMARKS OF CANCER AND THEIR CORRESPONDING MOLECULES IMPLICATED IN ORAL CARCINOGENESIS

Hanahan and Weinberg[7] first used the term "hallmarks of cancer" in 2000 to describe the fundamental biologic changes that characterize cancer cells, including the following: (1) sustaining proliferative signaling, (2) evading growth suppressors, (3) resisting cell death/apoptosis, (4) enabling replicative immortality, (5) inducing angiogenesis, and (6) activating invasion and metastasis. Subsequently, in 2011, the same authors, in the light of new data, completed the list of basic biological features of cancer, adding 2 more to the original 6 hallmarks: (7) deregulating cellular energetics and (8) avoiding immune destruction; furthermore, they described 2 additional features labeled enabling characteristics: (9) genome instability and mutation and (10) tumor promoting inflammation (Figure 2).[3] Advances in cellular and molecular biology have made it possible to recognize these basic features of cancer cells and use them for designing new targeting anticancer therapeutic approaches.[3,7]

However, it should be noted that although the hallmarks of cancer, as described by Hanahan and Weinberg,[7] have been widely accepted and extensively cited, constituting an "orthodoxy" in the field of contemporary cancer research, several other investigators have made important criticisms. Indeed, alternative views have been expressed suggesting that these

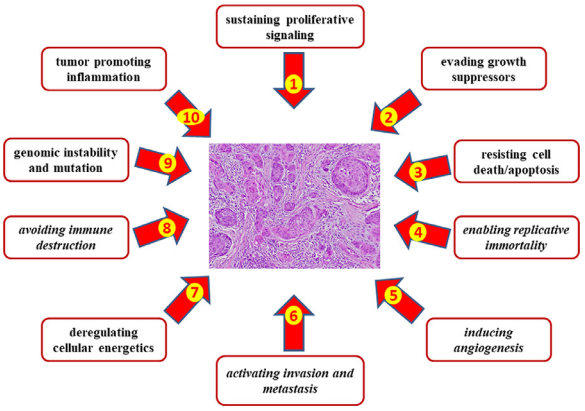

Fig. 2. According to Hanahan and Weinberg's seminal papers,[3,7] the fundamental biologic characteristics of cancer include the so-called hallmarks of cancer (1-8), as well as 2 enabling characteristics (9-10). Each of these broad, multifaceted, and intertwined biologic functions is under intensive investigation in oral squamous cell carcinoma, as related to its oncogenesis and progression.

hallmarks should be revisited and modified in light of new discoveries or realizations. For example, Fouad and Aanei[95] have critically reviewed the aforementioned 8 hallmarks (1-8) and 2 enabling characteristics (9-10) and have modified and reduced them to 7 (A-G): (A) selective growth and proliferative advantage (instead of 1 and 2), (B) altered stress response favoring overall survival (instead of 3 and 4, also encompassing 9), (C) vascularization (very similar to 5), (D) invasion and metastasis (identical to 6), (E) metabolic rewiring (similar to 7), (F) abetting microenvironment, and (G) immune modulation (similar to 8, also including 10). These authors have also redefined cancer hallmarks as "acquired evolutionary-advantageous characteristics that complementarily promote transformation of phenotypically normal cells into malignant ones, and promote progression of malignant cells while sacrificing/exploiting host tissue."[95]

Other authors have criticized not only the selection of specific hallmarks by Hanahan and Weinberg but even more the whole concept and value of these hallmarks. Specifically, Sonnenschein and Soto[96] have argued that the "hallmarks of cancer" approach, based on the precedent somatic mutation theory, is cell-based, follows a reductionist philosophy, and, more important, has failed to contribute to an increased understanding of the phenomenon under study (namely, cancer) or to provide a meaningful beneficial effect on cancer management and outcomes. Instead, these authors have put forward an alternative theory, the so-called tissue organization field theory, which is based on the premises that "proliferation and motility are the default state of all cells" and that "carcinogenesis is due to alterations on reciprocal

interactions among cells and between cells and their extracellular matrix."[96] Finally, other investigators have maintained an intermediate position, acknowledging the usefulness of the concept of hallmarks of cancer in providing an organizational framework for the mounting knowledge of cancer biology but also emphasizing that these hallmarks do not uniformly apply to all cancer cells at all times; rather, they should be considered components of a dynamic, multidimensional, and ever evolving network exhibiting spatial and temporal changes that account for the heterogeneity within any given malignant tumor and at least partly explain the difficulties of treating cancer cells with single targeted therapies.[97]

Notwithstanding the potential significance of these different philosophic approaches and theories in selecting the best strategy for studying, understanding, and, eventually, managing cancer, we prefer to selectively present the fundamental biologic features of oral cancer, largely following the traditional concepts of hallmarks of cancer, considering their prevailing place in the relevant literature in the last 2 decades.[9,12,42,98,99] Although a meticulous description of the molecular changes that occur during oral carcinogenesis contributing to the acquisition of the characteristics of malignancy is beyond the scope of this review article, a selective discussion of the major genetic and molecular changes that are mainly implicated in the development and progression of oral cancer will be attempted.

Deregulations in controlling cell cycle and proliferation

Deregulations in the control of cell cycle and proliferation constitute fundamental changes in the process of carcinogenesis.[8] To ensure the normal progression of the cell cycle, the proper function and regulation of specific checkpoints is required, allowing the temporary interruption of the cell cycle in order to confirm the presence of all prerequisites for its safe continuation. In this process, both positive and negative regulators are involved. Positive regulators, such as cyclins, cyclin-dependent kinases (CDKs), and various transcription factors (e.g., E2F and myc) promote the transition from one phase to the next.[11,100,101] On the contrary, negative regulators inhibit cell cycle progression, mainly including CDK inhibitors and tumor suppressor proteins pRb and p53. CDK inhibitors include the INK4 and Cip/Kip families, whose members (e.g., p15, p16, p21, p27) inactivate specific cyclin-CDK complexes.[11,100,101]

Alterations in the previously mentioned cell cycle regulators have been observed in both precancerous (dysplastic) lesions and oral cancer.[102] For example, a gradual increase in cyclin D1 levels has been reported in a significant percentage of precancerous and cancerous lesions of the mouth.[103] Also, loss of cell cycle control during the course of carcinogenesis leads to a disruption in cell proliferation biomarkers. Immunohistochemical investigation of the expression of Ki-67, PCNA, and MCM2 proliferative markers has revealed elevated levels in precancerous and cancerous lesions of the mouth.[103,104] Typically, it has been reported that the percentage of cells in the cell proliferation phase increases from 20% in the normal mucosa to about 45% in dysplastic lesions and as high as 60% in OSCC.[104] In addition, the detection of proliferative activity in the upper epithelial layers is an indication of extension of dysplastic features toward the surface because normally mitotic divisions are limited to the basal and parabasal layers of the epithelium.[42]

Evasion of apoptosis

Apoptosis or programmed cell death is an important function that ensures the elimination of cells that are no longer useful and/or are potentially harmful because of aging or unrepairable damage.[92] Under normal circumstances, apoptosis is controlled by proapoptotic and antiapoptotic factors. Dysfunction of apoptotic mechanisms that distort the balance between cell proliferation and death result in an uncontrolled increase in cell number and is considered a cornerstone of carcinogenesis.[105] Particularly in oral cancer, changes in the expression of various molecules that act as promoters or inhibitors of apoptosis, such as the Bcl-2 family proteins and survivin, are often detected.[92,106] It should be noted that even a modest increase in the levels of apoptotic activity of oral precancerous and cancerous lesions compared to the normal mucosa may not be sufficient to compensate for the increased cell proliferation. Therefore, the relative ratio between cell proliferation and apoptosis, and not their absolute changes in relation to the normal epithelium, is the determining factor that is disrupted during the course of carcinogenesis and leads to an increasing number of neoplastic cells.[92,106]

The role of tumor suppressor molecules p53 and pRb in the regulation of cell proliferation and apoptosis

Deregulation of the p53 tumor suppressor gene function is among the most common molecular phenomena in oral malignancies, as well as in cancer in general.[107,108] The main function of p53 is to pause the cell cycle to allow DNA repair or, in case of inability to repair the damage, to induce apoptosis. By doing so, p53 facilitates the elimination of cells that are at potential risk for malignant transformation. Conversely, inactivation of p53, as a result of various mechanisms, such as mutations, loss of heterozygosity, or MDM2-mediated degradation, deprives the cell of an important

regulatory mechanism to prevent carcinogenesis.[107,108] It is noteworthy that increased levels of immunohistochemical expression of p53 are observed in oral cancer, indicating the accumulation of a nonfunctional (mutated) form of the molecule.[42,109] Similarly, in oral precancerous lesions, it has been ascertained that p53 shows a progressive increase in expression levels and expansion into the upper layers of the dysplastic epithelium.[103] Loss of a p53 protective role, in combination with dysregulations in the expression of DNA repair−related molecules (DNA damage response), indicates the progressive loss of the cell's ability to control and eliminate DNA damage during the process of oral arcinogenesis.[93,110]

Retinoblastoma protein also exhibits a significant tumor suppressive activity, inhibiting the uncontrolled progression of the cell cycle. When active (i.e., in its unphosphorylated form), pRb binds to the transcription factor E2F, inhibiting its action and preventing the transition to the S phase of the cell cycle. On the contrary, inactivation of pRb due to phosphorylation by the cyclin D-CDK 4/6 complex releases E2F and allows continuation of the cell cycle.[111] The action of the cyclin D-CDK 4/6 complex is under the negative regulation of the p16 protein, which therefore exhibits tumor suppressive properties, similar to pRb. Deactivation of pRb or p16 (e.g., due to mutations) removes their tumor suppressive control over cell proliferation and may participate in oral carcinogenesis.[42,112,113]

Unlimited proliferation potential—immortalization

A key feature of cancer cells is their ability to multiply indefinitely without being subject to aging and programmed cell death. This property, described as immortalization, is directly related to the aberrant function of telomerase, a protein with enzymatic activity that helps maintain telomere length.[38,114] Because the normal reduction in telomere length in each cell division leads to cellular aging, exuberant activity of telomerase can cause cell immortalization, thus exerting oncogenic activity, as well as contributing to increased invasiveness.[38,115] Telomerase activation has been detected in oral cancer but also in precancerous lesions of the mouth, where it has been associated with an increased likelihood of malignant transformation.[116]

Angiogenesis

Angiogenesis, which refers to the formation of new vessels through proliferation, migration, and organization of endothelial cells, is a fundamental feature of malignant neoplasms and a necessary condition for their growth.[117] Tumors have developed mechanisms that induce angiogenesis, providing cancer cells with oxygen and nutrients, while at the same time facilitating their metastatic potential through ready access to the circulation. During carcinogenesis, an angiogenesis switch occurs, shifting the balance in favor of those factors that promote angiogenesis (pre-angiogenic) over those that inhibit it (anti-angiogenic); the end result of this switch is manifested by increased microvessel density.[118,119] This phenomenon occurs in OSCC, and it has also been observed in oral precancerous lesions.[120,121] The induction of vascular endothelial growth factor (VEGF) and nitric oxide synthase 2 seems to play a particularly important role in promoting angiogenesis by inducing endothelial cell proliferation and increasing the number and permeability of vessels in the tumor area.[42,122] For the latter, markers for angiogenesis have been extensively studied in oral cancer and associated with poor prognostic outcome and decreased survival.[123] In addition, VEGF has pleiotropic functions because it also displays anti-apoptotic properties and inhibits the host's immune response, thus facilitating the growth and metastatic spread of cancer.[11,124] Other important pre-angiogenic factors are angiopoietin 1 and 2, basic fibroblast growth factor, etc.[125,126] On the other hand, many factors with anti-angiogenic activity have been identified, such as angiostatin, endostatin, and others, whose expression levels and function are reduced in OSCC.[127]

Invasion and metastasis

The acquisition of invasive capability by epithelial cells of the oral mucosa, manifested by disruption of the basement membrane and expansion within the connective tissue, essentially marks the transition of a precancerous lesion to OSCC. Subsequently, cancer cells continue to invade the underlying tissue, such as the striated muscle fibers, nerve fibers, and bone. Eventually, they invade lymphatic or blood vessel walls, enter their lumina, and gain access to the circulation, which renders them capable of metastatic dissemination. In order for cancer cells to acquire invasive and metastatic properties, many successive changes occur in molecules that control cell adhesion and motility, such as cadherins, catenins, and integrins.[128,129] In addition, molecules that allow degradation of the extracellular matrix by enzymatic action, such as matrix metalloproteinases and cathepsins, are modified.[15,130,131] Of particular importance is the process of epithelial-mesenchymal transition through which epithelial cancer cells acquire morphologic and functional properties of mesenchymal cells, facilitating invasion and metastasis.[132] In turn, epithelial-mesenchymal transition is controlled and induced by molecular factors, such as TGF-β, Wnt, Notch, and others.[131]

The role of inflammation

The effects of inflammation in the process of carcinogenesis are 2-fold: it can participate in the destruction of cancer cells through immune surveillance (if this is functional at a sufficient level), and it can facilitate cancer spread and invasion through generation of growth signals and modification of the microenvironment.[133,134]

In head and neck cancer, elevated levels of inflammation-related molecules, such as the transcription factor NF-κB and cytokines (e.g., IL-6), are seen in neoplastic cells. NF-κB has a multifaceted activity because it promotes cell cycle development, angiogenesis, and invasion while inhibiting apoptosis.[135] NF-κB expression shows a gradual increase from the normal mucosa to precancerous and cancerous lesions.[136] On the other hand, IL-6, a major mediator of inflammation, exhibits elevated levels in various cancers, including OSCC.[86] Cyclooxygenases COX-1 and COX-2, key molecules in inflammatory processes, also show a gradual increase in expression from oral epithelial dysplasia to OSCC.[136] It is noteworthy that the chemopreventive activity of nonsteroidal anti-inflammatory drugs and COX inhibitors has been investigated in various cancers, including OSCC.[137]

Evasion of immune surveillance

Cancer cells develop mechanisms that allow them to escape recognition and destruction by the body's immune system.[4,138] These mechanisms, which are also functional in OSCC, include direct effects aiming to hamper antigen recognition on the surface of cancer cells (e.g., through structural changes in the molecules of the major histocompatibility complex I) or suppression of the immune cells, mainly cytotoxic T lymphocytes (e.g., by enhancing the expression of inhibitory molecules, such as cytotoxic T-lymphocyte-antigen 4 (CTLA-4), Programmed death protein 1 (PD-1) receptor and Programmed death-ligand 1 (PD-L1) ligand, and Fas Ligand (FasL) pro-apoptotic molecule).[90,139,140] In addition, OSCC cells acquire properties that render them resistant to the cytotoxic effect of T lymphocytes, while also indirectly affecting the function of the immune system through the secretion of various soluble molecular substances (such as VEGF, TGF-β, IL-6, IL-10, etc.).[140] In particular, CD8 + T lymphocytes, natural killer cells, and M1 macrophages have been shown to dysfunction in patients with OSCC.[141] In contrast, the activities of other cell types with immunosuppressive properties are enhanced, including regulatory T lymphocytes, myeloid-derived suppressor cells, and tumor-associated macrophages.[142,143]

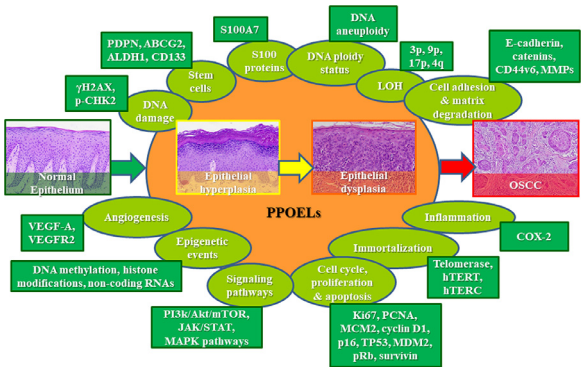

Fig. 3. A simplified schematic depiction of the major biologic functions disrupted in oral carcinogenesis (modified by Nikitakis et al.[12]). As shown in Figure 1, the transition from normal oral epithelium to oral precancerous lesions (also termed potentially premalignant oral epithelial lesions and histopathologically appearing as epithelial hyperplasia or dysplasia), and finally to invasive oral SCC, is driven by specific events and molecules (dark green boxes) affecting fundamental biologic processes (light green ovals). Noteworthy is that the sequence and combination of molecular aberrations differ from tumor to tumor and from patient to patient, emphasizing the need for individualized management of each case. *LOH*, loss of heterozygosity; *MAPK,* mitogen-activated protein kinase; *MMP*, matrix metalloproteinase; *mTOR*, mammalian target of rapamycin; *OSCC*, oral squamous cell carcinoma; *Rb*, retinoblastoma; *SCC*, squamous cell carcinoma; *VEGF*, vascular endothelial growth factor.

CONCLUSIONS

An ever-increasing understanding of the various molecular pathways disrupted during carcinogenesis, along with progressive elucidation of the oncogenic or tumor suppressive functions of a variety of genes and proteins, opens up promising horizons for the discovery and application of molecular markers. Figure 3 shows the main molecular functions that are disrupted during oral carcinogenesis and outlines the most important corresponding molecules that have been studied and implicated so far.[12] These advances have the potential to significantly improve the accurate diagnosis and prognostication of oral precancerous and cancerous lesions but also to serve as target molecules in individualized treatment protocols tailored to the specific molecular signature of each tumor.

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