Review Article

Epigenetics in oral squamous cell carcinoma

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Abstract

Oral squamous cell carcinoma (OSCC) is the most common type of oral neoplasm, accounting for over 90% of all oral malignancies and 38% of head and neck tumors. Worldwide, OSCC is the eighth most common human cancer, with more than 500,000 new cases being diagnosed every year with a fairly onerous prognosis, encouraging further research on factors that might modify disease outcome. Genetic and/or environmental risk factors associated with the development of oral cancer have been sufficiently understood (smoking, alcohol, betel, diet, living habits, etc.). Knowledge of the genetic basis in oral carcinogenesis is still a challenging task. To improve the diagnosis and prevention, a previously unknown type of chromatin modification, known as epigenetic, which is defined as heritable DNA changes that are not encoded in the sequence itself and which are reversible and increasingly appear to serve fundamental roles in cell differentiation and development are studied. Tumors shed their DNA into the blood and epigenetic changes that occur early during tumorigenesis, sometimes even in premalignant lesions, can provide valuable biomarkers. Key components involved in epigenetic regulation are DNA methylation, histone modifications and modifications in micro ribonucleic acids (miRNAs). Epigenetic modifications may contribute to aberrant epigenetic mechanisms seen in oral precancers and cancers. In the near future, epigenetic variations found in oral dysplastic cells can act as a molecular fingerprint for malignancies.

Keywords: Carcinogenesis, DNA, epigenetics, methylation, oral squamous cell carcinoma

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INTRODUCTION

Head and neck cancers constitute the sixth most common malignant tumors worldwide, affecting approximately 650,000 people and causing almost 350,000 cancer deaths per year.[1] Oral cancer is the most frequent cancer of head and neck, with squamous cell carcinoma being the commonest single entity, accounting alone for about 90% of all malignancies of the oral cavity.[2] Due to its related high mortality and low cure rate, oral squamous cell carcinoma (OSCC) represents a major public health problem, with a great individual and socioeconomic impact.

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Oral carcinogenesis is a multistep process modulated by endogenous and environmental factors. Among these, a major role is played by tobacco and alcohol regular intake as well as by human papillomavirus (HPV) persistent infection.[3,4] These predisposing factors may lead to a wide range of genetic and epigenetic events that promote genomic instability and tumor development and progression. The development and progression of oral premalignancy and OSCC are not only caused by irreversible changes in DNA sequence, including gene deletions, amplifications and mutations leading both to oncogenes activation or tumor suppressor genes (TSGs)

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inactivation but also by changes in gene expression that are not encoded in the DNA sequence and are designated as epigenetic changes.[5] Thus, chemical modifications in DNA and associated proteins can alter gene expression without affecting the DNA sequence.[6] Increasingly, new revelations about epigenetic modifications promise to transform all facets of cancer biology and to provide prophylactic, diagnostic and therapeutic benefits.

EPIGENETICS

The epigenetic changes refer to any heritable modifications in gene expression without alterations of the DNA sequence; they occur more frequently than gene mutations and may persist for the entire cell life and even for multiple generations.[7] Epigenetics literally means "above" or "on top of" genetics. It refers to external modifications to DNA that turn genes "on" or "off." These modifications do not change the DNA sequence, but instead, they affect how cells "read" genes.[8] In addition, epigenetic modifications are potentially reversible and transient. Epigenomics-based diagnostic tools for early cancer detection represent an exciting development.

Conrad Waddington, who coined the term, defined epigenetics as "the branch of biology that studies the causal interaction between genes and their product, which bring the phenotype into being."[9] Holliday described epigenetics as "the study of the mechanisms of temporal and spatial control of gene activity during the development of a complex."[10] According to Russo *et al*., epigenetics was defined as "the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in the DNA sequence."[11] The Greek prefix "*epi-*" in epigenetics means "on the top of" or "in addition to" genetics. In 2008, a consensual definition of epigenetics was established as a "stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence."[12] This article throws light on this seen but not so foreseen concept.

Tumors shed their DNA into the blood, and epigenetic changes that occur early during tumorigenesis, sometimes even in premalignant lesions, can provide valuable biomarkers. While the effects of genotoxic agents such as tobacco smoke and alcohol are the most important risk factors for the development of oral cancers, the interaction of epigenetic factors and genotoxic agents may synergistically increase the risk of oral carcinogenesis.

Three major types of epigenetic mechanism are currently known: DNA hypermethylation, histone modification and ribonucleic acid interference (RNAi). Disruption of any of these mutually reinforcing epigenetic mechanisms leads to inappropriate gene expression, resulting in cancer development and other "epigenetic diseases."[13]

EPIGENETIC MECHANISMS

DNA-methylation

DNA methylation is a heritable epigenetic mark involving the covalent transfer of a methyl group to the C-5 position of the cytosine ring of DNA by DNA methyltransferases (DNMTs). In plants, cytosines are methylated in both symmetrical (CG or CHG) or asymmetrical (CHH, where H is A, T or C) contexts. In mammals, DNA methylation occurs at cytosines in any context of the genome. DNA methylation is the most characterized type of chromatin modification. More than 98% of DNA methylation occurs in a CpG dinucleotide context in somatic cells while as much as a quarter of all methylation appears in a non-CpG context in embryonic stem cells. DNA methylation is regulated by a family of DNMTs: DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L [Figures 1 and 2].[14,15]

Most DNA methylation is essential for normal development, and it plays a very important role in a number of key processes including genomic imprinting, X-chromosome inactivation and suppression of repetitive element transcription and transposition and when dysregulated, contributes to diseases like cancer.[6]

CpG islands are often found hypermethylated in tumors, this event cause the transcriptional "silencing" of TSGs contributing to cancer progression; on the contrary, it has also been described the derepression of proto-oncogenes transcription by hypo/demethylation, this leading to increased mutation rates and to chromosome instability, which constitutes an early hallmark of tumor cells.[16-18] The genes most frequently hypermethylated and silenced in cancer cells reside in chromosome regions commonly show loss of heterozygosity (LOH). The LOH of hypermethylated genes is often involved in metastatic ability and in tumor neoangiogenesis.[19]

DNA METHYLATION IN ORAL CARCINOGENESIS

The genes found hypermethylated in OSCC, includes cell-cycle control (p16, p15), apoptosis (p14, death-associated protein kinase [DAPK], p73 and Ras association family [RASSF] 1A), Wntsignalling (APC, WIF1 and RUNX3), cell–cell adhesion (E-cadherin) and DNA-repair (MGMT and hMLH1) genes.[20,21]

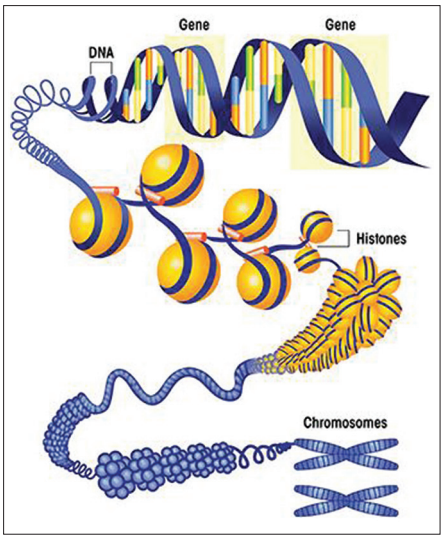

Figure 1: Chromosome structure. DNA is tightly wound around proteins called histones and packaged into cells' nuclei in the form of chromosomes. Genes are sections of DNA that, under the right circumstances, can be transcribed into proteins[62]

CDKN2A

CDKN2A gene maps on chromosome 9p21 and encodes the cell-cycle regulatory protein p16, which inhibits the cyclin-dependent kinase 4 and 6 activity, inducing cell-cycle arrest in the G1 phase. Hypermethylation of p16 ranges from 23% to 76% in OSCC are reported.[22,23]

E-CADHERIN AND N-CADHERIN

CDH1 gene (cadherin 1 type 1) is located on chromosome 16q22.1 and encodes for E-cadherin, a 120-kd single-span transmembrane glycoprotein, with five extracellular and one cytoplasmic domain, interacting with catenins. This molecule is mainly involved in the formation of adhesive junctions in epithelial cells, playing a fundamental role in formation and maintenance of intercellular adhesion, cell polarity, intracellular signaling and tissue architecture. E-cadherin absence is strictly linked to alterations in cell key functions and motility. In addition, it is shown that the loss of its expression is frequently involved in tissue metastasis. It was shown a correlation between the low expression of E-cadherin and a more aggressive behavior of OSCC E-cadherin gene hypermethylation frequency ranged between 7% and 46%.[24,25]

Vered *et al*. analyzed the recent literature evaluating the immunoexpression of E-cadherin in OSCC and stressed the need for a critical review of the immunohistochemistry-based expression evaluation of this molecule, to better define the association between its expression and clinical outcome.[26] Tumors with high

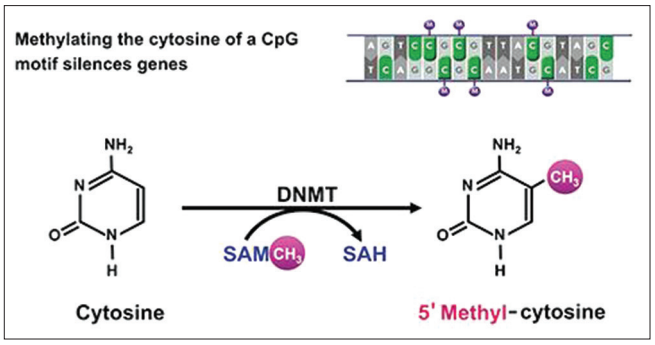

Figure 2: DNA methylation[63]

N-cadherin value were characterized by a more aggressive behavior. These data suggested that N-cadherin could have a potential role in predicting the biological behavior of SCC.[27]

Phosphatase and tensin homolog deleted on chromosome 10

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a tumor-suppressor gene located on chromosome 10q23.3, the loss of which expression is thought to be involved in important cellular processes including survival, differentiation, proliferation, apoptosis and invasion. In addition, due to lack of control of the signaling pathways that mediate apoptosis and migration, such as Ras/phosphoinositide 3-kinase (PI3K)/Akt, it plays a fundamental role in tumor cell survival and proliferation and metastasis. PTEN is frequently deficient in several malignancies because of mutations or epigenetic changes. In addition, evidence has also been provided supporting that CpG islands of the PTEN promoter are methylated in several types of human cancers, such as endometrial carcinoma, gastric nonsmall-cell lung carcinoma and cervical cancer.[28-31]

In a recent review, Díez-Pérez *et al*. report the data relative to a comparison study between oral cancer tissue and normal mucosa, showing a 77.8% reduction of gene expression, due to its promoter methylation.[32]

p53

TP53 gene maps on chromosome 17p13.1 and encode a tumor suppressor protein, also called p53, involves in many fundamental cell processes, such as cell-cycle progression, cellular differentiation, DNA-repair and apoptosis. When an endogenous or exogenous stress occurs, p53 levels increase and lead to block cell cycle, allowing the DNA-repair. Loss of p53 function alters the ability of cells to respond to stress or damage, leading to genomic instability. p53 is mutated in the majority of human cancers, including oral cancers, with frequency ranging from 25% to

69%.[33,34] In several instances, however, p53 shows a loss of function due to epigenetic events, instead of genetic alterations.[35]

Death-associated protein kinase 1

DAPK1 gene maps on chromosome 9q34.1, it encodes a pro-apoptotic calcium/calmodulin regulated serine/threonine kinase-inducing apoptosis (p53-dependent apoptotic checkpoint). The reported frequency of DAPK promoter hypermethylation ranges from 18% to 27%.[36,37]

MGMT

MGMT gene is located on chromosome 10q26, it encodes MGMT (06-methylguanine-DNA methyl transferase), a DNA repair enzyme that removes adducts caused by alkylating agents; such DNA repair activity favors the resistance of cells to treatment-induced apoptosis. Silencing this gene allows alkylated guanine to accumulate, restoring apoptosis. The frequency of hypermethylation in OSCC ranges from 7% to 68%.[36]

Retinoic acid receptor B2

Retinoic acid receptor B2 gene (*RARB2*) is a TSG belonging to the RARB family, mapping on chromosome 3p24. It is frequently inactivated in cancer, mainly by methylation. It is linked with the deregulation of cell proliferation in tumors as well as in preneoplastic lesions. Promoter methylation of the *RARB2* gene was generally reported in cancers of the head and neck region (67%) and in a significant percentage of precancerous lesions (>50%).[38]

Ras association family 1 and Ras association family 2

RASSF1 (3p21.3) and RASSF2 (20p13) belong to the RASSF of proteins involved in the Ras/PI3K/Akt pathways. Huang *et al.* showed that in almost the 50% of patients treated with radiotherapy Ras/PI3K/Akt pathways were activated in association with RASSF1A/RASSF2A gene silencing through promoter methylation. In addition, Imai *et al.* found RASSF2 methylated in 26% of OSCC evaluated.[39,40]

Hypermethylation of p14ARK, p16INK4a, p15, MGMT, DAPK, GSTP1, RARB and p53 have been found in dysplasia and in histologically normal-appearing margin of OSCC resection. Thus, methylation could be considered as an early promising marker of malignant progression.[35] Less is known on the presence and role of hypomethylation: To date, it has been reported only the possible occurrence of secreted frizzled-related protein 1 (SFRP1) (8p11.21) hypomethylation in OSCC, but the data are too few to achieve an overview of the phenomenon in these tumors. Contrary to Sogabe *et al.*, who observed that SFRP1 together with SFRP2 and SFRP5 were methylated in OSCC, Pannone *et al.* found SFRP1 significantly demethylated in cancer ($P < 0.05$).[41]

Ribonucleic acid interference

In 2006, Andrew Fire and Craig C. Mello shared the Nobel Prize in Physiology or Medicine for their work on RNAi in the nematode worm *Caenorhabditis elegans*, which they published in 1998. RNAi is a system involved in controlling gene activation in living cells. Two types of small RNA molecules – miRNA and small interfering RNA (siRNA) – are considered as key mechanisms related to RNAi.[42]

Small interfering ribonucleic acid

siRNA, sometimes referred to as short interfering RNAs or silencing RNAs, represents a class of double-stranded RNA molecules, 20–25 nucleotides in length, that play a notable role not only in the RNAi pathway, where it interferes with the expression of a specific gene, but also in RNAi-related pathways as well as in antiviral mechanism or in shaping the chromatin structure of a genome. siRNAs were first discovered by David Baulcombe's group at the Sainsbury Laboratory in Norwich, England, as part of posttranscriptional gene silencing in plants. This discovery led to a surge in interest in harnessing siRNA for biomedical research and drug development. It is expected that in some situations turning off or knocking down the activity of a gene with an siRNA could produce a therapeutic benefit.[43]

Micro ribonucleic acid

miRNAs are a recently discovered class of noncoding endogenous small RNAs,[44] which have a crucial role in the control of gene expression and are associated with promotion and progression of malignancies.[45]

They are involved in many fundamental cellular processes such as proliferation, development, differentiation and apoptosis in normal and neoplastic cells, where they are referred to as oncomiRs (oncogenic miRNA). They act as mediators of epigenetic gene regulation, by interacting with mRNA, either by inhibiting mRNA translation or causing mRNA degradation. Recent studies have been shown that miRNAs act as putative tumor suppressors and may also undergo epigenetic silencing in cancer.[46]

Cervigne *et al.* examined miRNA (miR) expression changes in 43 sequential progressive oral leukoplakia samples from 12 patients and 4 nonprogressive leukoplakias from 4 different patients. The findings were validated using quantitative reverse transcription polymerase chain reaction in an independent cohort of 52 progressive dysplasias and OSCCs and 5 nonprogressive dysplasias.

Global miR expression profiles distinguished progressive leukoplakia/OSCC from nonprogressive leukoplakias/normal tissues. Of 109 miRs, which were highly expressed exclusively in progressive leukoplakia and invasive OSCC, miR21, miR181b and miR345 expression was consistently increased and associated with increases in lesion severity during progression. The authors hypothesized that overexpression of miR21, miR181b and miR345 may play an important role in malignant transformation.[47]

Wong *et al*. found that the level of miR-133a and miR-133B was significantly decreased in OSCC when compared with normal epithelia samples. These low levels led to the activation of a potential oncogene pyruvate kinase type M2.[48]

Kozaki *et al*. investigated the miR-137 and miR-193a expression levels alteration in some OSCC cell lines, demonstrating that the epigenetic silencing of both miRNA, caused by DNA hypermethylation, could have a key function in oral cancer progression [Table 1].[49]

Hu *et al*. demonstrated that miR-504 plays an important role during carcinogenesis, acting as a negative regulator of p53. The potential role of miR-504 as new diagnostic, prognostic and therapeutic tools has been recently discussed by Hu *et al*. and by Gorennchtein *et al*., both hypothesizing a clinic advantage in OSCC patient management.[50]

CHROMATIN DYNAMICS AND HISTONES MODIFICATIONS

The chromatin structure is highly regulated by complex interactions between many molecular pathways that influence normal and tumor cell fate, histones and chromatin modifiers mainly induce the changes of chromatin architecture. Histones have a structural role in the chromatin architecture entering into the constitution of nucleosomes. Acetylation, methylation, phosphorylation and ubiquitination are major histone modifications, combination of which may constitute the "histone code" that extends and modulates the genetic code.[51,52]

Table 1: Micro ribonucleic acids whose expression is either upregulated or deregulated in oral squamous cell carcinoma[50]

Cellular function	miRNAs	Expression in OSCC
Proliferation and apoptosis	miR-137, miR-193a, miR-133a, miR-133b, miR-503, miR-15a	Down regulated
Metastasis	miR-21, miR-24, miR-184, miR-222 and miR-138, miR-211 and miR-31, miR-21	Upregulated Downregulated Upregulated Downregulated
Chemoresistance	miR-23a, miR-214, miR-98	Upregulated

miRNAs: Micro ribonucleic acid, OSCC: Oral squamous cell carcinoma

Among molecules that regulate the chromatin assembly, histone chaperones play an essential role. They drive histones incorporation into newly synthesized or remodeled chromatin.[53]

In this process, the chromatin assembly factor-1 (CAF-1) exerts a pivotal role: it destabilizes heterochromatic structures during replication, allowing the replication machinery to progress through heterochromatin. CAF-1 is a protein complex, formed of three subunits with different molecular weight (p48, p60 and p150) and delivers histones H3 and H4 on newly synthesized DNA during DNA replication and DNA repair. CAF-1 facilitates the incorporation and assembly into chromatin of H3K56 acetylated histones, in response to oxidative stress and DNA damage and also contributes to resolve the mismatch-containing strands, restoring chromatin structure on the completion of double strand break repair.[54]

The histone acetylation status mainly depends on the activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Some recent works evaluated the role of HDAC inhibitors in OSCC cell lines and OSCC tissues. Increase in histone acetylation generally correlates with gene activation and results from the dynamic interplay between HATs and HDACs. The ultimate mediators of histone methylation-associated gene silencing appear to be proteins that bind specific modified histone and recruit effector protein complexes.[55]

Among these, the ones which seem to play a significant role in carcinogenesis belongs to ING protein family. The ING proteins (ING 1-5) are involved in cell cycle, apoptosis and senescence. The ING family emerged as putative TSG and its major mechanism of activity entails the conserved plant homeodomain, which binds to histones in a methylation-sensitive pathway. In cancer cells, INGs mRNA levels are often lost or suppressed, but their genes are rarely mutated; indeed, the inactivation of the normal function is achieved through the allelic loss of genomic regions containing the ING gene, alteration in the ING promoter region, variation of mRNA splicing efficacy or reduced mRNA stability. Recently, the potential roles of ING proteins as prognostic biomarkers, detector of aggressive behavior of tumors as well as predictive factor of chemoradiotherapy response, have been hypothesized.[56]

HUMAN PAPILLOMAVIRUS

High risk human papillomavirus (HR-HPV) persistent infection has been recently indicated as an independent risk

factor for head and neck cancers and recent literature has documented the link between HR-HPV persistent infection of oral epithelium and the development of OSCC.[57,58]

The association between HR-HPV persistent infection of oral epithelium and the development of OSCC characterizes a distinct subgroup of malignancy arisen in a younger and higher socioeconomic group, often nonsmokers/nondrinkers.[59]

This subset of HR-HPV positive lesions shows different genetic and molecular profile and has been associated with a more favorable prognosis and significantly lower malignant progression rate and a better responsiveness to chemo- and radio-therapy when compared with HPV-negative ones although more often they are poorly differentiated.[60]

However, it is not yet well known if the better biological behavior and the better response to the adjuvant therapies of the HR-HPV-associated OSCC might be due to the indirect action of innate or acquired cofactors, moreover to formally confirm the role of HPV as an etiological agent of OSCC, additional evidence is required.[60]

EPIGENETICS AND ENVIRONMENT EXPOSURE

Chronic exposure of human mucosal epithelial cells to tobacco-derived carcinogens drives hypermethylation of several TSGs. The reactive oxygen species associated with chronic inflammation is another source of DNA damage and whereas cigarette smoke causes hypomethylation.

Cadmium: Interact with the methyltransferase DNA binding domain-interference in enzyme-DNA interaction-reduces genome methylation.

Arsenic: Detoxification of as is by enzymatic methylation using SAM-depressed SAM levels can cause global DNA hypomethylation.

Nickel: Replace magnesium in DNA interactions, enhance chromatin condensation and trigger *de novo* DNA methylation-leading to the inactivation of the gene. In addition, increases global H3K9 mono- and demethylation, a/w increased DNA methylation and long-term gene silencing. Chromium: Reduce *in vitro* H3 phosphorylation and trimethylation and acetylation marks in H3 and H4– a/w lung cancers.[61]

Folate, Vitamin B-12, methionine, choline (soymilk, broccoli) and betaine (wheat bran, spinach, sweet potato, beef, etc.) can affect DNA methylation and histone methylation through altering 1-carbon metabolism. Pantothenic acid is a part of CoA to form acetyl-CoA, which is the source of acetyl group in histone acetylation. Genistein (soyabean, coffee) and tea catechin affects DNMT Resveratrol (grape, blueberry, raspberry, mulberry), butyrate (released by gut bacteria), sulforaphane (broccoli) and diallyl sulfide (garlic and onion) inhibit HDAC and curcumin inhibits HAT.[62]

EPIGENETIC THERAPY

The potential reversibility of epigenetic states offers opportunities for novel cancer drugs that can reactivate epigenetically silenced tumor-suppressor genes. Blocking either DNA methyltransferase or HDAC activity could potentially inhibit or reverse the process of epigenetic silencing.[63]

DNA methyltransferases and HDACs are the two major drug targets for epigenetic inhibition to date although others are expected to be added in the future. The network of multiple reinforcing interactions involved in epigenetic silencing suggests that combination therapy would be a particularly appropriate strategy to achieve clinical efficacy. The use of epigenetic inhibitors in association with traditional anticancer therapeutic agents looks very promising as a tool to improve the chemosensitivity of OSCC.[61]

FUTURE TRENDS

Epigenetic changes in oral mucosa cells emerge as a warning light of ongoing/occurred malignant transformation, concurring to outline a "molecular fingerprint" which can be very helpful in the diagnosis of malignancies.

Epigenetic mechanisms have emerged as important contributors in the pathogenesis of various human cancers. More importantly, since these alterations occur frequently in the process and are potentially reversible, this makes them ideal for exploitation as disease biomarkers as well as therapeutic targets in human cancer. It is just a matter of time before most of these approaches are put into everyday clinical practice, which will undoubtedly help reduce oral cancer morbidity and mortality in the not-so-distant future.

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There are no conflicts of interest.

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