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Epigenetic mechanisms in oral carcinogenesis

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Abstract

Dysregulation of gene expression is a frequent occurrence in oral squamous cell carcinoma (OSCC). However, accumulating evidence suggests that in contrast to genetics, epigenetic modifications consisting of aberrant DNA methylation, histone modifications and altered expression of miRNAs induce OSCC tumorigenesis and perhaps play a more central role in the evolution and progression of this disease. The unifying theme among these three epigenetic mechanisms remains the same, which is aberrant regulation of gene expression. In this article, we provide a comprehensive review of the impact of epigenetics on oral tumorigenesis with a systematic report on aberrant DNA methylation, histone modifications and miRNA regulation in the pathogenesis of OSCC. We provide insights into recent studies on the prospect of biomarkers for early detection and indication of disease recurrence, and novel treatment modalities.

Keywords

DNA methylation; epigenetics; genomic instability; histone modification; miRNA; oral cancer

Each year more than 100,000 patients die of oral squamous cell carcinoma (OSCC) worldwide [1]. OSCC is a multifactorial disease in which chronic tobacco and alcohol use constitute two major risk factors, while chronic inflammation, viral infections (human papillomavirus), betel quid chewing and genetic predisposition are supplementary factors that contribute towards its pathogenesis. In order to improve survival by means of enhanced prevention and therapeutic options, it is of the essence to understand the basic molecular mechanisms driving oral tumorigenesis. Besides the limited and somewhat poorly understood role of genetic alterations in the pathogenesis of OSCC, accumulating evidence over the last decade highlights that epigenetic alterations, consisting of aberrant DNA methylation, histone modifications and altered expression of miRNAs, may actually play a much larger role in the initiation and progression of OSCC. An accumulating body of data suggests that the two major risk factors, smoking and alcohol consumption, have a direct

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impact on the dynamic regulation of gene expression orchestrated by epigenetic mechanisms.

Epigenetic mechanisms may influence dysregulation of gene expression in a number of different ways. Aberrant promoter hypermethylation can interfere with the binding of transcription factors to the DNA of tumor suppressor genes, resulting in transcriptional silencing of key genes involved in protection of cells from the process of unregulated growth. By contrast, global hypomethylation may cause reactivation of methylation-silenced proto-oncogenes as well as leading to enhanced genomic instability. Post-translational histone modifications (e.g., acetylation or methylation of histone residues) cause conformational changes of the DNA tertiary structure, thereby either enhancing or blocking the binding of transcription factors to promoter DNA. Finally, miRNAs regulate gene expression on a post-transcriptional level by inhibiting protein translation through degradation or repression of the mRNA transcript.

In the last decade, epigenetics has become the major focus of numerous investigations, as it provides previously unrecognized mechanistic insights into the etiology of OSCC genesis. This review article provides a comprehensive and an up-to-date description of the current state of epigenetic research, with full reporting of aberrant DNA methylation, histone modifications and effects on miRNA expression in OSCC tumors. This includes all pertinent articles that were accessible through a PubMed search that was performed at the time of compilation of this article in May 2012. The keywords used for literature search included ‘oral cancer methylation’, ‘oral cancer histone acetylation OR oral cancer histone deacetylation OR oral cancer histone modification’ and ‘oral cancer miRNA’.

DNA methylation in oral cancer

Both genome-wide hypomethylation and promoter hypermethylation play a critical role in cancer development. Figure 1 illustrates the process of OSCC tumorigenesis and highlights the molecular alterations that accrue during the progression of this malignant disease. There is an increased recognition that global DNA hypomethylation contributes to tumorigenesis by multiple potential mechanisms. First, through the reduction of methylation at DNA repetitive elements (e.g., LINE-1, Alu sequences), which are distributed throughout the human genome. Demethylation at these methylated repeat elements results in enhanced chromosomal instability. Second, by accidental demethylation of some of the evolutionarily conserved and methylation-silenced promoter regions of various endoparasitic elements and proto-oncogenes, which can lead to their reactivation and contribute towards accelerated carcinogenesis. Third, DNA methylation alterations may possibly contribute to oral carcinogenesis by loss of imprinting. In such a scenario, hypomethylation may result in reactivation of naturally methylated silent, imprinted bialleles and alter gene expression. This mechanism has been discussed in numerous different tumor entities including head and neck squamous cell carcinoma; however, it has not been investigated in OSCC as yet.

On the other hand, aberrant promoter hypermethylation is another key player in OSCC tumorigenesis. Aberrant methylation in this instance occurs at the CpG-rich sequences (or CpG islands) present within the promoter regions of many tumor suppressor genes. Hypermethylation of promoter CpG dinucleotides results in a closed chromatin configuration (or heterochromatin), which blocks access for transcription factors to bind to the promoter region of tumor suppressor genes, resulting in their transcriptional silencing. This process of aberrant hypermethylation of 5-methyl-cytosines at CpG dinucleotides is catalyzed by DNA methyltransferases (DNMTs). Given the growing awareness for the aberrant methylation in human cancer, DNMT inhibitors, aimed at reactivating potentially

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methylation-silenced tumor suppressor genes, are now being explored as potential treatment choices for OSCC.

Unequivocal evidence indicates that DNA methylation is closely related to OSCC tumorigenesis. Analysis of DNA extracted from OSCC tissues and oral premalignant lesions (OPLs) found they exhibit more frequent and higher levels of DNA methylation compared with healthy or corresponding normal tissue from neoplastic tissues [2]. The use of tobacco, which is a major risk factor for the development of OSCC, has been linked to nonspecific global hypomethylation [3,4]. In contrast to smokers, patients who drink heavily have an increased risk for CpG hypermethylation of multiple OSCC-related genes. Chronic inflammation of the oral mucosa is another risk factor that can potentially modify the methylation status of various genes in OSCC tumors [5]. The occurrence of multiple CpG methylation sites in a panel of tumor-related genes in OSCC was highly associated with cancer stage and may correlate with lymph node metastasis [6]. Abundant investigations have focused on gene-specific aberrant DNA methylation in OSCC tumors. This systematic review covers all investigations on DNA methylation in oral cancer as displayed in Table 1 (for a more comprehensive list of aberrantly methylated genes, please refer to Supplementary Table 1; see online at www.futuremedicine.com/doi/full/10.2217/fon.12.138). To date, most oral cancer-related publications focused on CpG methylation of *APC, Survivin, E-cadherin, MGMT, MLH1, p14*^{ARF}*, p15*^{INK4B}*, p16*^{INK4A}*, RAR*β and *RASSF* genes. Hence, these genes were selected for a more detailed discussion below.

Adenomatous polyposis coli

APC (adenomatous polyposis coli) is a tumor suppressor gene that indirectly inhibits cell proliferation through the WNT-1 (wingless) signaling pathway in human cancer cells. *APC* gene methylation and its associated gene silencing occur early in the development of oral carcinogenesis, by allowing increased β-catenin target gene expression, which results in increased cell division [7]. Hypermethylation of the *APC* promoter was detected in 15% of OSCC tumor tissues and 25% of OSCC cell lines [7,8]. Thus, hypermethylation of the *APC* promoter may be an important step in the dysregulation of the WNT signaling pathway, leading to an increase in cell proliferation during carcinogenesis.

Survivin/BIRC5

The function of Survivin, or BIRC5 (baculo-viral inhibitor of apoptosis repeat-containing protein 5), is to increase tumor cell numbers by promoting cell proliferation and preventing cell apoptosis. There is no evidence for hypermethylation of *Survivin* in OSCC tissues [9]. This oncogene is ordinarily methylated (silenced) in normal tissues, but is frequently upregulated in OSCC due to its hypomethylation. Thus, over-expression of *Survivin* due to hypomethylation leads to an increase in cell proliferation with a concomitant decrease in cell death promoting carcinogenesis. In one study, increased *Survivin* expression was associated with a more aggressive and invasive tumor phenotype [10]. Using the hamster buccal pouch mucosa as an experimental model for oral carcinogenesis, Chen *et al.* observed that mineral oil-treated animals had normal *Survivin* methylated alleles, while the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA)-treated animals had buccal pouch carcinomas with a hypomethylated *Survivin* allele [11,12]. The results of these studies indicate that hypomethylation of the *Survivin* gene appears to be an important step in the process of OSCC carcinogenesis.

CDH1/E-cadherin

Hypermethylation of the *CDH1* (cadherin 1) gene, which encodes the adhesion protein E-cadherin, is highly associated with OSCC and may be an early event in oral carcinogenesis [13]. *CDH1* promoter hypermethylation in OSCC tumors is associated with invasive tumor

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behavior and a worse prognosis [14,15]. Interestingly, hypermethylation and loss of heterozygosity of the *APC* gene have also been linked with a change in the cytoplasmic expression pattern of E-cadherin [8]. However, results of *CDH1* methylation studies have been inconsistent. Several studies reported the frequency of *CDH1* methylation in OSCC tissues as ranging from as little as 17% to as high as 85% [6,14,16,17]. Basaloid cells of dysplastic lesions were reported to exhibit increased *CDH1* hypermethylation [18], while two studies did not detect any significant difference in methylation status between OSCC and healthy control tissues [16,17]. The variability in results of these studies indicates that *CDH1* methylation may not serve as a good marker for OSCC.

O-6-methylguanine-DNA methyltransferase

The DNA repair gene *MGMT* (O-6-methylguanine-DNA methyltransferase) is important in preventing carcinogenesis, since it removes mutagenic adducts from O(6)-alkyl-guanine in DNA. Silencing *MGMT* via methylation is thought to be an early event in carcinogenesis [19], as 75% of OSCC tissues demonstrate *MGMT* gene silencing via hypermethylation of its promoter region [20]. It has been postulated that CpG methylation may be only one way to silence *MGMT* expression, as additional factors may also modulate its gene expression [21]. For example, betel quid chewing is a known risk factor for development of OSCC, and patients who had chewed betel quid demonstrated a lack of *MGMT* expression [20]. Hypermethylation of the *MGMT* promoter is associated with poorer survival in patients with OSCC [22]. Multiple independent studies evaluated OSCC tissues and found that 12–74% had *MGMT* methylation [6,16,19,22,23]. Based upon these studies, it is apparent that methylation of the *MGMT* gene in OSCC tumors may be useful both as a diagnostic tool, and possibly as a predictor of patient survival.

mutL homolog 1

Owing to the significant role of the DNA mismatch repair gene *MLH1* (mutL homolog 1) in prevention of the accumulation of mutations in DNA, it is not surprising that it is a frequent target of epigenetic modifications in many human cancers. Hypermethylation of its 5′-promoter region is believed to silence *MLH1* gene expression and, hence, downregulate protein expression. *MLH1* methylation may be associated with a higher risk for development of oral malignancies [24], and in a study of *MLH1* methylation status and OSCC, 76% of OSCC tissues showed *MLH1* promoter methylation compared with 0% of the control tissues [25]. Interestingly, most of the *MLH1*-methylated promoters in OSCC samples corresponded to early clinical stages, with only a few late-stage OSCCs demonstrating *MLH1* promoter methylation [25]. This observation of a lack of CpG methylation of *MLH1* in healthy tissue or tumor-adjacent normal tissue has been subsequently validated in other studies [25,26]. Overall, methylation of *MLH1* in OSCC tissue is observed in up to 76% of the examined cases [24–26], indicating it may serve as a biomarker for the early presence of OSCC.

p14^{ARF}

p14^{ARF} is a tumor-suppressor gene that not only works to control cell proliferation and division, but was also recently discovered to regulate tumor-induced angiogenesis [27]. Hypermethylation of *p14^{ARF}* results in loss of p53 function and deactivation of p21-induced cell proliferation. *p14^{ARF}* hypermethylation, a rather late event in carcinogenesis, is associated with increased tumor size and tumor stage, and nodal metastasis [23]. It has been shown that *p14^{ARF}* hypermethylation in late-stage tumors is associated with a lower recurrence rate and a better clinical outcome compared with patients with tumors that were not *p14^{ARF}*-hypermethylated [28]. While neither healthy tissue nor tumor-adjacent tissues exhibited any signs of *p14^{ARF}* methylation, a small proportion (4%) of the OPLs demonstrated *p14^{ARF}* hypermethylation [29]. In studies of OSCC tumors, 14 –44% of the

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tumors had hypermethylation of the *p14*^{ARF} promoter [23,28]. In a study focusing on betel quid-related OSCC, *p14*^{ARF} hypermethylation was frequently detected in OPLs, suggesting it could serve as a prognostic marker for early detection of betel quid-associated OSCC [29]. The fact that the majority of the hypermethylation of *p14*^{ARF} promoters was observed in dysplasia or early stages of OSCC may denote it is a transient early event in the process of carcinogenesis in OSCC.

p15^{INK4B}

p15^{INK4B} is a tumor suppressor gene that acts to inhibit cell growth by preventing cell cycle progression during the G1 phase. Hypermethylation and gene inactivation of the *p15*^{INK4B} checkpoint control gene are commonly observed in OPLs and OSCC [29]. While healthy and tumor-adjacent tissues did not typically exhibit signs of methylation for *p15*^{INK4B} [29], 9–28% of the examined OSCC tissues revealed promoter hypermethylation of this gene [30]. Taken together, these reports indicate that aberrant methylation of the *p15*^{INK4B} gene may serve as a marker for OSCC.

p16^{INK4A}

The product of the *p16*^{INK4A} gene is another cell cycle inhibitor, and the *p16*^{INK4A} gene was one of the first genes investigated for the role of methylation in oral cancer. *p16*^{INK4A} gene expression is highly regulated by hypermethylation of its promoter, as well as heterozygous deletion of its chromosomal locus [31]. Hypermethylation of the *p16*^{INK4A} gene is frequently detected in corresponding normal mucosa of OSCC tissues, which is thought to contribute to the risk of the ‘field defect’ concept during the early stages of OSCC [19]. In line with these results, three individual studies showed that 20–58% of the OPLs have methylated *p16*^{INK4A} promoters [32–34], and the risk for progression to OSCC was generally greater in patients with *p16*^{INK4A}-methylated OPLs [32]. *p16*^{INK4A} hypermethylation is associated with increased tumor size and stage (stages III and IV), nodal metastasis, increased risk for disease recurrence and a poor prognosis [13,23,28]. When OSCC tissues were tested for the presence of *p16*^{INK4A} methylation, 12–88% of the OSCC cases revealed evidence for aberrant methylation of this tumor suppressor gene [6,19,22,23,28,35–37]. Besides the biological variation in *p16*^{INK4A} methylation among various studies, these results are also dependent upon samples and methodology used in different laboratories [38,39]. Two animal studies examined methylation of *p16*^{INK4A} in OSCC versus normal tissues. In the first study, a hamster buccal pouch mucosa experiment demonstrated aberrant *p16*^{INK4A} promoter methylation in 26% of all DMBA-treated animals [40]. In another study using the rat tongue model to study dysplasia induced by 4-nitroquinoline 1-oxide, it was noted that *p16*^{INK4A} methylation increased with the degree of dysplasia, and this observation was inversely correlated with the expression of this gene in dysplastic tissues [41]. These results suggest that *p16*^{INK4A} promoter methylation may serve as a good prognostic indicator for the development of OSCC.

Retinoic acid receptor β

*RAR*β (retinoic acid receptor β) is a member of the thyroid-steroid hormone receptor superfamily of nuclear transcription factors associated with cell growth and differentiation. There has been much interest in the role of *RAR*β promoter methylation due to the use of retinoids as chemopreventive agents in OSCC; however, the role of *RAR*β in OSCC remains unclear. A high frequency of *RAR*β promoter methylation (73%) has been observed in an OSCC tissue pyrosequencing study, although 62% of aberrant methylation has been noted in adjacent normal tissues [17]. In an examination of OPLs, over half (53%) of the cases had methylated *RAR*β promoters [42]. Based upon these results, it is unclear whether methylation of *RAR*β is a very early step in OSCC carcinogenesis, or whether it simply represents a generalized aberration across all oral mucosal tissues.

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Ras-association domain family 2

There are ten *RASSF* (Ras-association domain family) gene members, which have a variety of important cell functions. *RASSF* proteins function as tumor suppressors and regulators of the cell cycle, apoptosis, and microtubule formation. In recent years, it has been recognized that gene expression of several members of this family can be controlled by their aberrant methylation. In a study of *RASSF2*, 39% of OSCC tissues were methylated in at least one *RASSF2* gene [43], while another investigation of OSCC tissues reported 22% methylation of the *RASSF1A* gene and 28% for the *RASSF2A* gene [44]. Interestingly, simultaneous methylation of both the *RASSF1A* and *RASSF2A* genes was associated with a poor disease-free survival [44]. Other trials have reproduced these results and demonstrated up to 12–38% methylation of the *RASSF1* gene in OSCC tissues [6,22,44]. In one study of betel quid-associated OSCC, 93% of the OSCC tissues demonstrated methylation of the *RASSF1* gene, suggesting a high association of *RASSF1* methylation in tumors due to betel nut chewing [45]. Thus, epigenetic silencing of the *RASSF* family of genes via methylation may play an important role, not only in the induction, but also in the outcome, of patients with OSCC.

DNA methylation alterations as diagnostic biomarkers in OSCC

Accumulating evidence suggests that early detection of OSCC is perhaps one of the most promising approaches for the treatment of this deadly malignancy. Tumor tissues certainly have a very high diagnostic value; however, patient's compliance for additional biopsies is rather low and the surgical method is quite invasive for frequent screening. By contrast, saliva provides an unlimited, easily available specimen resource for the early detection of OSCC in high-risk patients, and is suitable for monitoring tumor recurrence. Thus, screening a set of methylated cancer-related genes in the saliva or oral rinses of patients can provide an attractive panel of biomarkers for the early detection of OSCC. Accordingly, in a quest to identify and develop robust and preferably noninvasive biomarkers, Viet and Schmidt performed a methylation array analysis for detection of 807 cancer-associated genes comparing the saliva of healthy subjects with that of OSCC patients, preoperative and postoperative saliva and OSCC tissue of cancer patients [46]. This elegant study identified 34 genes that were highly methylated in tumor tissues and preoperative saliva samples, while there was no evidence for methylation of these CpG loci in the postoperative saliva of OSCC patients and healthy subjects [46]. With the help of an array-based analysis interrogating 27,578 CpG sites, Guerrero-Preston *et al.* tested OSCC saliva samples for their methylation status. Compared with saliva of healthy subjects, the saliva of OSCC patients showed significant differences in *HOXA9* and *NID2* methylation. The genes *HOXA9*, *NID2* or a combination of *HOXA9* and *NID2* have therefore been suggested as biomarkers due to high sensitivity of 75, 87 and 50% and high specificity of 53, 21 and 90%, respectively [47]. Another clinical oral rinse study by Nagata *et al.* investigated the methylation status of a panel of tumor-related genes to develop a noninvasive method of detection for OSCC. In a comparison of oral rinse samples from OSCC and control patients, aberrant methylation was detected in a total of 13 genes; eight of these genes demonstrated higher levels of methylation in OSCC versus control washings. Upon further evaluation, a combination of *ECAD*, *TMEFF2* and *MGMT* methylation offered a very high sensitivity (97%) and specificity (92%) for detecting OSCC[48].

The *LINE-1* (long interspersed nuclear element) gene is used as a surrogate marker for global methylation levels in human cancers. Even though the sequence of each *LINE* element is homologous, *LINE-1* methylation levels may differ at various loci. In OSCC cell lines, 28–39% of CpG sites occur within *LINE-1* repeat elements [5]. Analyses of oral rinses and the corresponding OSCC tissues of cancer patients both revealed *LINE-1* hypomethylation [49]. Compared with oral tissue from healthy subjects, OSCC tissues demonstrated *LINE-1* hypomethylation independent of tumor stage, tumor site, histological

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grading or risk factors. In addition to hypermethylated gene loci, *LINE-1* hypomethylation may therefore be an excellent biomarker for use in noninvasive early OSCC detection [49].

Several studies have suggested $p16^{INK4A}$ may be used as a biomarker to predict malignant transformation, as hypermethylation of this gene is associated with metastasis and overall poor survival in OSCC patients [37]. While healthy normal tissues do not show any signs of $p16^{INK4A}$ hypermethylation, normal tissues from smokers without cancer often demonstrate methylation of this gene [50], suggesting that $p16^{INK4A}$ hypermethylation may be a very early event in OSCC development [50]. In patients with leukoplakia, an oral precancerous condition, methylation of $p16^{INK4A}$ was observed in the oral rinses of 44% of patients, and hypermethylation increased with stage of the disease [51]. In one longitudinal clinical study investigating the prognostic usefulness of methylation biomarkers in the malignant transformation of OPL, hypermethylation of the $p16^{INK4A}$ gene correlated with malignant progression [52]. In addition to predicting malignant transformation, $p16^{INK4A}$ hypermethylation was shown to predict local OSCC recurrence [53]. To identify a patient's individual risk for OSCC recurrence, it may be useful to evaluate more than just the $p16^{INK4A}$ methylation status of their tumor tissue. One study assessed the effectiveness of testing for $p16^{INK4A}$ promoter hypermethylation in the tumor tissues and in the surgical margins. Promoter hypermethylation of $p16^{INK4A}$ was observed in 87% of the tongue squamous cell carcinoma (TSCC) tissues. But, most importantly, patients with histologically tumor-free and $p16^{INK4A}$-hypermethylated surgical margins had a sixfold increased risk for local recurrence compared with patients with histologically tumor-free and negative margins [54]. In another report, $p16^{INK4A}$ promoter hypermethylation was detected in 65% of the OSCC tissues, 55% of which also revealed evidence for its methylation in the matching serum DNA. Interestingly, 75% of the patients with $p16^{INK4A}$-hypermethylation both in tumor tissue and serum were affected by tumor recurrence [55]. Thus, monitoring patients with precancerous lesions or patients after OSCC treatment via $p16^{INK4A}$ hypermethylation may be a valuable tool in identifying malignancy or malignant recurrence in OSCC patients.

In addition to histological examination of the surgical specimens, the detection of promoter hypermethylation in cancer-free surgical margins or in oral rinses/scrapings may be a simple and noninvasive approach for predicting future OSCC recurrence or malignant transformation. For instance, hypermethylation of genes such as $p16^{INK4A}$ and *MGMT* is significantly associated with poor overall survival, which can facilitate establishment of individualized treatment plans in OSCC patients [6]. In summary, the discovery and development of epigenetic biomarkers provides us with not only more attractive and practically feasible cancer screening modalities, but also equips us with molecular tools that can guide personalized treatment decision-making in the clinics in the not so distant future.

DNA methylation inhibitors as therapeutic agents in OSCC

As mentioned previously, CpG methylation alterations may also provide an avenue for establishing epigenetic therapeutic regimens for this malignancy. For instance, treatment of OSCC cells (HSC-3) with the DNMT inhibitor zebularine alone resulted in tumor growth inhibition, as evidenced by decreased cell growth and reduction in the number of cells accumulated in the G2/M cell cycle phase [56]. While the molecular mechanisms for these observations remain unclear at this time, it was intriguing to observe that when zebularine was used as an adjuvant to cisplatin or 5-fluoro-uracil chemotherapy, it greatly enhanced the apoptotic activity of the cisplatin treatment, but it decreased the efficacy of treatment with 5-fluorouracil[57].

MGMT is another gene involved in OSCC whose expression is modulated via abnormal methylation. OSCC cell lines with low levels of *MGMT* methylation responded with a greater antiproliferative response to 5-fluorouracil chemotherapy, compared with cell lines

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with high levels of *MGMT* methylation [58]. However, when cells were pretreated with O6-benzylguanine (O6-BG), a potent inhibitor of *MGMT*, the anti-proliferative effect of 5-fluorouracil was much greater in these nonresponsive cells in comparison with mock-treated cell lines [58]. Therefore, one must be cautious when considering combination chemotherapies and inhibitors for use in the treatment of OSCC.

Green tea extracts are thought to prevent malignant transformation of precancerous lesions to OSCC. Overall, the clinical response rate tended to be better in patients treated with green tea extracts versus placebo, and there was a significantly greater achievable response rate with the higher doses of extracts (750 or 1000 mg/m^2) compared with placebo groups [59]. In studies with OSCC cell lines, green tea extracts suppressed cancer cell invasion by reversing hypermethylation of the *RECK* gene, resulting in its enhanced mRNA expression [60]. In summary, data from these preliminary experiments clearly favor the enthusiasm and excitement for the development of safe and effective DNMT inhibitors that can be used alone or in conjunction with other chemotherapeutic regimens for better management of patients with oral cancer.

Histone modifications in oral cancer

Post-translational modifications of histones are frequently observed in oral cancer. These epigenetic alterations occur primarily at the N-terminal tails within each of the four histone complexes (H3, H4, H2A and H2B). Various modifications include methylation, acetylation, ADP-ribosylation, phosphorylation, ubiquitination and sumoylation of specific residues within these histone tails. These processes are generally reversible and modify the tertiary DNA structure. Alterations at histone tails have a direct impact on chromatin condensation, which can exist either in a heterochromatin or euchromatin configuration. The heterochromatin form is frequently observed in neoplastic cells, during which the DNA is coiled up into a closed, compacted chromatin configuration, making it impossible for transcription factors to access DNA and allow active gene transcription. By contrast, euchromatin configuration is a theme in healthy, normal cells, in which DNA is present in an open configuration, allowing transcription factors easier access to DNA. Histone modifications are tightly associated with DNA methylation, particularly in the context of tumor suppressor gene silencing, leading to an enhanced rate of tumorigenesis.

Methylation of specific lysine residues within histone tails is associated with either activation or transcriptional repression of gene transcription. However, this effect is primarily dependent upon the position of the specific lysine residue being methylated, as well as the number of sites methylated [61]. Histones can be methylated at only one or all of the six lysine residues within the histone tails H3 (K4, K9, K27, K36 and K79) and H4 (K20). Histone methylation at H3K9, H3K27 and/or H4K20 is generally associated with repressive histone marks that lead to transcriptional silencing of the corresponding gene [62], while methylation of H3K4, H3K36 and H3K79 is associated with activating histone marks that allow active transcription of the involved gene [63]. Histone methylation can occur in a mono- (me1), di- (me2) or tri-methylated (me3) form on various lysine residues.

The processes of DNA methylation and histone methylation are tightly coregulated and collectively play an important role in carcinogenesis. In one study, the patterns of DNA and histone methylation were positively correlated in normal, OPL and OSCC tissues [64]. In another study, the pattern of H3K4 histone methylation was associated with OSCC malignancy. The frequency of transcriptionally inactive, H3K4me2 histones was greater in OSCC compared with normal tissues, while the converse was true for activating H3K4me3 modifications [65]. A similar pattern of histone methylation was found in OPLs, such as leukoplakia, compared with normal tissues. Leukoplakia tissues presented themselves more

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like OSCC tissues with regards to their pattern of histone methylation. The results of these investigations help reinforce the notion that in many instances, leukoplakia should be considered a premalignant condition for the development of OSCC and treated accordingly. Thus, changes in H3K4 histone methylation may occur as an early event during OSCC carcinogenesis [65].

Hypomethylation of H3K9, rather than hypermethylation, is associated with transcriptional activation due to the establishment of loosely configured euchromatin. When an OSCC cell line was treated with ornithine decarboxylase antizyme-1, it induced hypomethylation of H3K9me2, as well as changes in the levels of a number of DMNTs. These results indicate that hypomethylation of histones may be a potent means for accelerating OSCC tumorigenesis, which causes enhanced DNA instability, loss of cell cycle control and decreased ability for DNA repair [62].

Similar to histone methylation, histone deacetylation, catalyzed by various histone deacetylases (HDACs), plays a significant role during oral carcinogenesis. It has been demonstrated that HDAC2 improves the stability of the HIF-1α protein, which may lead to enhanced invasion and migration in OSCC [66]. Likewise, expression of HDAC6 was upregulated in OSCC and was found to be stage-specific; the higher the stage, the greater the activity [67]. The authors concluded that HDAC6 expression may be important in determining tumor aggressiveness in oral cancers. In addition to deacetylating histones, HDAC6 was revealed to be capable of deacetylating α-tubulin as well, thus promoting microtubule-dependent cell motility, a process that may be important to the development of metastasis [68].

Interestingly, Arif *et al.* discovered that histone H3, primarily H3K14, is hyperacetylated in OSCC [69]. These investigators discovered that in the KB oral cancer cell line, increased H3 acetylation was nitric oxide-dependent. Since nitric oxide is produced during the inflammatory process, which has been linked to the initiation and development of OSCC, this is a particularly important finding[69]. Furthermore, hydrazinocurcumin, a potential therapeutic drug, was able to inhibit histone acetyltransferase activity and reduce oral tumor growth in a xenograft mouse model [69]. The results of these experiments demonstrate an important mechanistic role for hyperacetylation of H3K14 in OSCC pathogenesis.

The poly(ADP-ribose) polymerase (PARP) family of enzymes is responsible for the post-translational covalent transfer of ADP-ribose to proteins, as well as formation of polymers of poly(ADP-ribose). The activity of PARP-1, DNA synthesis rates and the degree of ADP-ribosylation is greatest in actively proliferating OSCC [70], a finding that is ascribed to histones rather than nonhistone chromosomal proteins. The addition of poly(ADP-ribose) to histones loosens the chromatin structure. This nucleosome modification facilitates DNA repair through large protein complexes such as chromatin assembly factor (CAF)-1. CAF-1 is a molecular chaperone consisting of three subunits (p48, p60 and p150). Its role is the integration of H3K56-acetylated histones into the chromatin. The nuclear expression of CAF-1/p60 is upregulated in multiple tumor entities including OSCC. Worst prognosis in terms of survival and metastasizing behavior may be predicted in PARP-1^{high} and CAF-1/$p60^{high}$ OSCC tissues [71]. Concurrent CAF-1-mediated deregulation of cell proliferation as well as DNA repair also occurs in aggressive TSCC. An immunohistochemical evaluation reported that CAF-1/p60 is commonly expressed in TSCC tissues, whereas CAF-1/p150 may be downregulated. Both parameters are associated with poor clinical outcome and worse prognosis [72]. Taken together, DNA methylation, acetylation and/or poly(ADP)-ribosylation may all be important factors in the development of OSCC.

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Histone modifications as diagnostic biomarkers in OSCC

In contrast with advancements made with regards to identification of DNA methylation alterations in OSCC, noninvasive measurement of histone modifications still remains a concept in its infancy. Overexpression of HDAC2 in OSCC tissue was identified to be associated with advanced tumor stage, tumor size, metastasis and significantly shorter overall survival, and may therefore make a good biomarker [73]. A very recent pilot study determined the feasibility of using DNA collected from oral rinse samples to perform chromatin immunoprecipitation assays to analyze the interaction of DNA methylation, histone modifications and gene expression. The authors provided very encouraging data showing the epigenetic control of $p16^{INK4A}$/*CDKN2A* gene could be determined in the DNA from oral rinses. The positive results obtained in this study have provided a springboard to embark upon future studies using oral rinse samples to investigate patterns of histone modifications in OSCC[74]. In 1993, Das investigated ADP-ribosylation in OSCC tissues from two different tumor stages, along with the analysis of normal adjacent oral tissues. The activity of poly(ADP-ribose) synthetase was reported to increase during oral carcinogenesis. With increasing malignancy, a progressive increase in ADP-ribosylation of histones was noticed in purified nuclei of OSCC tissue, suggesting that ADP-ribosylation may be a potential marker for OSCC [70].

HDAC inhibitors as therapeutic agents in OSCC

Based upon the recognition for the role of histone modifications in human cancer, there is growing interest in treating OSCC patients with a variety of epigenetic inhibitors, more specifically using a panel of different inhibitors of HDAC activity. There are 18 assorted HDACs that can be divided into four distinct classes (Class I–IV) based on their sequence identity and function. The various classes of HDAC inhibitors currently available are summarized in Table 2. Interestingly, they have varied affinities for the different classes of HDACs. HDAC inhibitors represent a novel, promising compilation of antineoplastic compounds that inhibit deacetylation of histones and thereby act to promote the uncoiling of chromatin allowing activation of a variety of genes associated with the regulation of cell survival, proliferation, differentiation and apoptosis. HDAC inhibitors may enable the re-expression of silenced tumor suppressor genes in OSCC and thereby reverse the malignant phenotype. When combined with well-established chemotherapeutics, HDAC inhibitors synergistically enhance the efficacy of conventional chemotherapy [75]. HDAC inhibitors fall into the category of prospective therapeutics for a variety of diseases, perhaps most importantly for their application in cancer therapy. In 2006, the US FDA approved the HDAC inhibitor suberoylanilide hydroxamic acid for use as a therapeutic agent for advanced cutaneous T-cell lymphoma. At present, several novel HDAC inhibitors are undergoing clinical trials and may soon be launched, in combination with more traditional chemotherapies, as therapy to treat cancer [76].

Trichostatin A—The hydroxamic acid trichostatin A (TSA) was one of the earliest HDAC inhibitors to be discovered. TSA and other hydroxymates are the only known inhibitors of the classical HDACs (Class I, II and IV). TSA owes its pharmacological effect to zinc ion complexation [77]. TSA was shown to inhibit proliferation in oral cancer cells (YD-10B) by causing cell cycle arrest at the G2/M phase [78]. Additionally, TSA induced apoptosis in these cells by enhancing $p21^{WAF1}$ expression, decreasing Cyclin B1 and blocking the phosphorylation of Cdc2 [78]. In another study examining OSCC cell lines (HSC-4, Ho-1-N-1 and Ho-1-U-1), similar results were obtained with TSA with regards to inhibiting cell growth and inducing apoptosis in all three OSCC cell lines and inducing expression of $p21^{WAF1}$ protein expression [79]. Additionally, TSA enhanced the protein expression of p21, cyclin E, cyclin A, Bak and Bax, while it simultaneously inhibited the expression of HDAC1, p53, E2F-1, E2F-4 and hyperphosphorylated Rb [79]. Analogous results were

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found using TSCC cells (TCA8113), in which TSA inhibited cell growth by inducing a G2/M arrest and induced apoptosis through upregulation of the apoptosis-inducing protein Bax [80]. This study also showed that the NF-κB pathway is involved in these effects, as TSA blocked NF-κB activation, which in turn caused a decrease in the levels of the anti-apoptotic proteins Bcl-2 and Bcl-xL [80]. Even though TSA has these many effects in blocking the proliferation of cancer cells, its use as an anticancer agent is limited due to its high toxicity.

Butyric acid derivatives—The butyric acid derivatives represent another well-investigated category of HDAC inhibitors. Phenylbutyrate, a prominent butyric acid derivative, promotes DNA repair and survival in healthy cells. Phenylbutyrate treatment has been shown to reduce oxidative stress, TNF-α expression and the incidence of severe oral mucositis [81]. As an adjuvant to radiotherapy, it lowered the risk of OSCC and tumor progression in hamster cheek pouches [81]. Sodium butyrate, another butyric acid derivative, inhibited OSCC proliferation and induced G1 and G2/M cell cycle arrest *in vitro* in multiple human OSCC cell lines, including HSC-3, HSC-4, SCC-1 and SCC-9 [82,83]. In concert with inhibiting cell cycle progression, sodium butyrate induced expression of G1 phase cell cycle regulatory proteins CDK6, $p21^{WAF-1}$ and p27, while decreasing the expression of the S/G2 phase protein CDK2 as well as phosphorylation of Rb. The HDAC inhibitor sodium butyrate caused Cyclin D1 upregulation and Cyclin B1 and Cyclin E downregulation [83]. However, even though phenylbutyrates and sodium butyrates are generally viewed as rather weak HDAC inhibitors [77], they have been entered into clinical trials for study, particularly for their effects on myelodysplastic disorders.

In addition, other novel HDAC inhibitors have been described in the field of oral cancer. (S)-HDAC42 is a phenylbutyrate-based inhibitor that is potent in suppressing tumor growth in a number of cancers. It acts through histone acetylase-dependent and -independent pathways. Using OSCC tumor cell lines, it was observed to have a more pronounced antiproliferative effect compared with suberoylanilide hydroxamic acid [84].

Romidepsin (FR901228/FK228)—Romidepsin is a naturally occurring depsipeptide that is a potent inhibitor of Class I HDACs [77]. Analogous to butyric acid derivatives, romidepsin induced cell cycle arrest at G1 and G2/M [85]. *In vitro* studies targeting the role of mapsin, a proposed tumor suppressor gene, discovered that addition of romidepsin to OSCC cells caused a time-dependent re-expression of mapsin transcripts [86,87]. These results suggested that reinduction of mapsin expression after romidepsin treatment may help to explain the recovery of normal biological functions such as blockage of cell invasion and tumor angiogenesis. In another study using OSCC cell lines, it was shown that expression of the cell senescence regulating gene hTERT (telomerase reverse transcriptase) increased after treatment with romidepsin [86,87]. Since mapsin and hTERT both may be important factors in regulating cell mortality and invasion, targeted treatment with the HDAC inhibitor romidepsin to induce their expression may be an important addition to cancer therapy. Besides these, there are currently a whole host of HDAC inhibitors being developed and tested for their antitumor activities [88].

HDAC inhibitors as adjuvant chemotherapeutic agents—Numerous studies have revealed the synergistic behavior of the HDAC inhibitors with well-established chemotherapeutics such as cisplatin and 5-fluorouracil; hence, these agents have been suggested as adjuvants to chemotherapy as well as radiotherapy [75]. The synthetic benzamide HDAC inhibitor MS-275 enhanced the cytotoxic effectiveness as adjuvant to cisplatin in the treatment of OSCC [75,77], while the combination of low-dose cisplatin and suberoylanilide hydroxamic acid displayed a synergistic effect in inducing greater cytotoxicity and apoptosis than cisplatin treatment alone. As an adjuvant to chemotherapy, suberoylanilide hydroxamic acid may enhance OSCC cell sensitivity, demonstrating a much

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greater effect in the cell cycle fractions from the G0/G1 phase and G1/S phase [75,89,90]. In addition, suberoylanilide hydroxamic acid enhances treatment efficiency in combination with 5-fluorouracil [56]. The results of all of these experiments indicate that HDAC inhibitors have an important use in sensitizing oral cancer cells to the effects of chemo- and radiotherapy.

miRNAs in oral cancer

miRNAs are the most recent entrants into the category of epigenetic gene expression regulators. miRNAs represent small RNA molecules with important regulatory functions, wherein each miRNA has the potential to target multiple mRNAs or gene targets. miRNAs regulate gene expression on a post-transcriptional level, inhibiting protein formation by degradation or repression of translation of the mRNA transcript. As the function and role of miRNAs are still a fairly new field of research, the studies associated with miRNA regulation of OSCC are recent, but increasing rapidly. To date, 1898 unique mature human miRNAs have been identified. An overview of the known OSCC-associated miRNAs is depicted in Figure 2 and Table 3 (for a more detailed list of individual studies on miRNA expression patterns in OSCC please refer to Supplementary Table 2).

miRNA expression patterns in TSCC—In studies of oral cancer, researchers have looked for specific miRNA signatures or profiles in oral cancer tumors. Wong *et al.* performed miRNA profiling by examining the expression level of 156 miRNAs in TSCC and compared the expression with normal tissues. Using a threefold expression difference as a cutoff threshold, this group identified 24 miRNAs that were overexpressed in TSCC (*miR-17-5p, miR-21, miR-30a-3p, miR-31, miR-34b, miR-34c, miR-104, miR-124a, miR-124b, miR-128a, miR-132, miR-134, miR-137, miR-147, miR-154, miR-155, miR-181c, miR-184, miR-197, miR-198, miR-213, miR-325, miR-338* and *miR-372*) while 13 miRNAs were underexpressed (*miR-26b, miR-99a, miR-100, miR-107, miR-125b, miR-133a, miR-133b, miR-138, miR-139, miR-149, miR-194, miR-195* and *miR-219*) [91]. The number of miRNAs modulated in TSCC may signify the importance of dysregulated expression of these specific miRNAs in this disease.

A microarray-based miRNA profiling analysis identified that *miR-21* is frequently overexpressed in TSCC compared with adjacent normal mucosa, and high *miR-21* expression is associated with poor prognosis in TSCC by virtue of inhibiting apoptosis [92]. Likewise, upregulation of *miR-24* [93] and *miR-184* [94] in TSCC is associated with enhanced proliferation and reduced apoptosis [93]. By contrast, reduced *miR-138* expression correlates with enhanced risk for metastasis in TSCC, potentially through regulation of *RhoC* and *ROCK2* Rho GTPases [95]. Our understanding of the functions of these miRNAs may help us better understand their role in TSCC.

miRNA expression patterns in OSCC—Identification of a miRNA signature that could determine the risk for malignant transformation of OPL would be an extremely useful tool for the early detection of OSCC. In a comparison of miRNA profiles from leuoplakia with and without progression, 109 miRNAs were found to be differentially expressed only in progressive leukoplakia and invasive OSCC [96]. However, most interestingly was that increased expression of *miR-21, miR-181b* and *miR-345* was associated with an increased severity of OPL, suggesting these miR-NAs may serve as biomarkers for the early identification of progressive leukoplakias that are at risk for progressing into malignant lesions [96].

While examining the miRNA profiles in 18 OSCC cell lines compared with the immortalized oral keratinocyte cell line RT7, a panel of 148 miRNAs were analyzed [97]. A

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total of 54 miRNAs (37%) were underexpressed in OSCC cell lines compared with control cells. Interestingly, it was also noted that the down-regulated expression of *miR-34b, miR-137, miR-193a* and *miR-203* was a consequence of CpG hypermethylation; a finding that further reiterated the intimate association between different epigenetic mechanisms in human carcinogenesis. These *in vitro* results were validated in primary OSCC tissues, wherein downregulation of the tumor suppressors *miR-137* and *miR-193a* via epigenetic silencing was a more frequent occurrence than the reduced expression of *miR-34b* and *miR-203* [97]. Thus, the interrelationship between miRNA expression and other epigenetic mechanisms have the potential to be extremely important in the process of carcinogenesis.

In an OSCC animal model, six Syrian hamsters were treated with the carcinogen DMBA, followed by miRNA microarray analysis to investigate the expression profiles of miRNAs during the development of oral carcinogenesis. While five miRNAs (*miR-21, miR-200b, miR-221, miR-338* and *miR-762*) were over-expressed, 12 miR-NAs (*miR-16, miR-26a, miR-29a, miR-124a, miR-125b, miR-126-5p, miR-143, miR-145, miR-148b, miR-155, miR-199a* and *miR-203*) were downregulated due to this oncogenic stimulus [98]. Overexpression of *miR-21* has subsequently been validated in other studies, and it has been proposed that *miR-21* downregulates the expression of the tumor suppressor gene *PDCD4*, which is associated with nodal metastasis and invasive potential in OSCC [99].

While the list of miRNAs that are differentially expressed in oral cancers is continuously evolving, it has been shown that *miR-211,* which is mapped to 15q13, is frequently overexpressed, and is associated with tumor progression, nodal metastasis, vascular invasion and poor prognosis of OSCC [100]. On the other hand, *miR-133a* and *miR-133b* are downregulated in OSCC. This promotes oral carcinogenesis through the pathway of inducing proliferation and inhibition of apoptosis, by permitting overexpression of pyruvate kinase type M2, a potential oncogene in solid tumors [101]. The mechanistic details underlying differential regulation of miRNAs remain poorly understood. In a recent study, it was shown that dicer, an RNase III endo-nuclease responsible for miRNA maturation, exhibited aberrantly high protein expression in OSCC cell lines and tissues. Other reports indicate the *let-7* family of miRNAs might regulate the expression of dicer post-transcriptionally in oral carcinogenesis [102].

These data clearly support the idea that aberrant expression of miRNAs may have a causal link with OSCC pathogenesis and, once these expression patterns are more firmly established, some of these may serve as important diagnostic, prognostic and predictive biomarkers in oral carcinogenesis.

miRNA alterations as diagnostic biomarkers in OSCC

Selected studies have thus far explored the potential of miRNAs as biomarkers for the early detection of oral neoplasias. Given the abundance of miRNA alterations in human cancer and their stability in a wide variety of bodily fluids and tissues, it is not surprising that miRNA detection may become a valuable tool for OSCC diagnostics in tumor tissues, plasma and/or saliva obtained from OSCC patients.

Plasma levels of *miR-31* were suggested to serve as tumor biomarkers for early OSCC detection and indicators for recurrence [103]. Plasma *miR-31* was not only increased in OSCC patients compared with healthy controls, but its levels also significantly dropped in patients following surgical removal of the tumor [103]. On similar lines, *miR-184* was frequently overexpressed in pre-operative plasma samples from TSCC patients, while the postoperative samples demonstrated a significant drop after successful removal of the primary tumor [91,94]; a concept highlighting the usefulness of this miRNA as an indicator of tumor recurrence. Furthermore, increased expression of *miR-10b* was observed in plasma

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samples from patients with OSCC and premalignant OPLs compared with normal subjects, suggesting its usefulness as an early diagnostic tool for identification of OSCC[104].

While analyzing saliva samples from OSCC patients, Park *et al.* found that *miR-125a* and *miR-200a* are significantly underexpressed in OSCC patients compared with healthy controls [105]. Oral rinses obtained from OSCC patients and healthy subjects displayed aberrant *miR-200c* and *miR-141* methylation and aberrant *miR-375* and *miR-200a* expression [106]. Collectively, there is accumulating evidence that highlights not merely the feasibility, but potential superiority of miRNA-based biomarkers for the earlier diagnosis and prognosis of patients with oral cancers.

Conclusion & future perspective

Epigenetic mechanisms have clearly 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. In terms of oral cancer, the data thus far have been from somewhat preliminary experiments; nonetheless, the results are exciting, highlighting the involvement of epigenetic mechanisms in every stage of oral carcinogenesis. Given the promising results, future research should concentrate on a more focused approach that can reap the benefits of existing data for the earlier diagnosis of OSCC. Additionally, it can better appreciate the possible role of epigenetic drugs as possible adjuvants to chemo- and radiotherapy. It is just a matter of time before most of these approaches are put into everyday clinical practice, which will undoubtedly help reduce OSCC morbidity and mortality in the not-so-distant future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Executive summary

Epigenetics of oral cancer

- Regulation of gene expression may be modified by three epigenetic mechanisms: aberrant DNA methylation, histone modifications and expression of miRNAs.

DNA methylation in oral cancer

- Aberrant promoter hypermethylation occurs at CpG sites within gene promoters leading to impeded access for transcription factors binding to the DNA, and eventually resulting in transcriptional silencing of key growth regulatory tumor suppressor genes.
- Global hypomethylation of CpG dinucleotides within the body of the gene may invoke enhanced genomic instability by permitting reactivation of evolutionarily methylation-silenced proto-oncogenes and retrotransposons.

Histone modifications in oral cancer

- Post-translational histone modifications (e.g., acetylation or methylation of histone residues) cause conformational changes of the tertiary DNA structure in such a manner that it inhibits transcription factor binding to DNA and results in transcriptional gene silencing.

miRNAs in oral cancer

- miRNAs regulate gene expression on a post-transcriptional level, either by inhibiting protein translation through degradation or by repression of translation of the mRNA transcript.

Future perspective

- Detection of aberrant gene expression is possible from tissue, plasma or saliva providing a rationale for the development of noninvasive screening biomarkers for oral squamous cell carcinoma.
- Epigenetic therapy, including the use of DNA methyltransferases and histone deacetylase inhibitors, is gaining attention. Specific and safe epigenetic drugs are identified that have the potential for a more effective and personalized medicine for oral malignancies.

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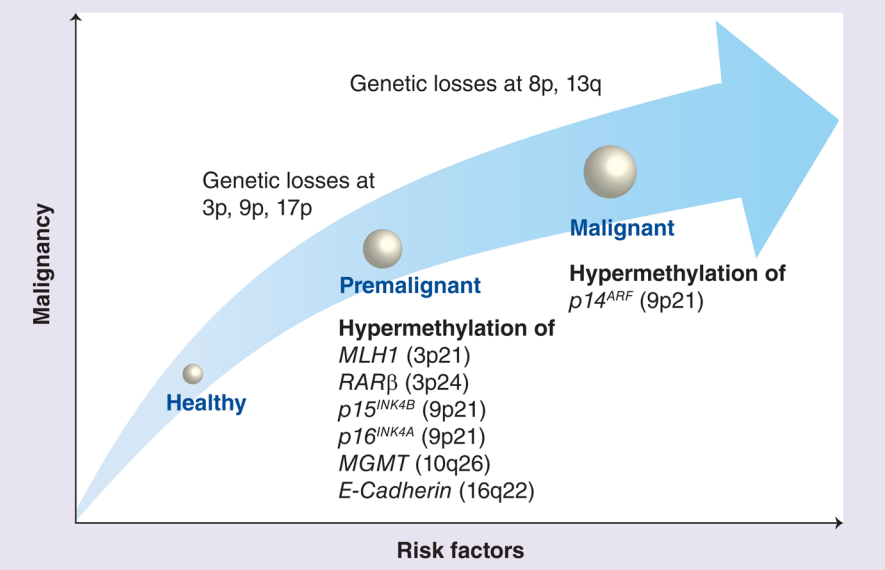

Figure 1. Progression of oral squamous cell carcinoma

While oral squamous cell carcinoma progresses from one stage to the next, the oral tissue is marked by distinct molecular alterations during each step. Risk factors for oral cancers, such as alcohol consumption or tobacco abuse, lead to a series of genetic and epigenetic alterations in the oral tissues, which eventually may progress to advanced oral cancer. This figure highlights some of the specific genetic and aberrant methylation alterations that occur during oral squamous cell carcinoma progression.

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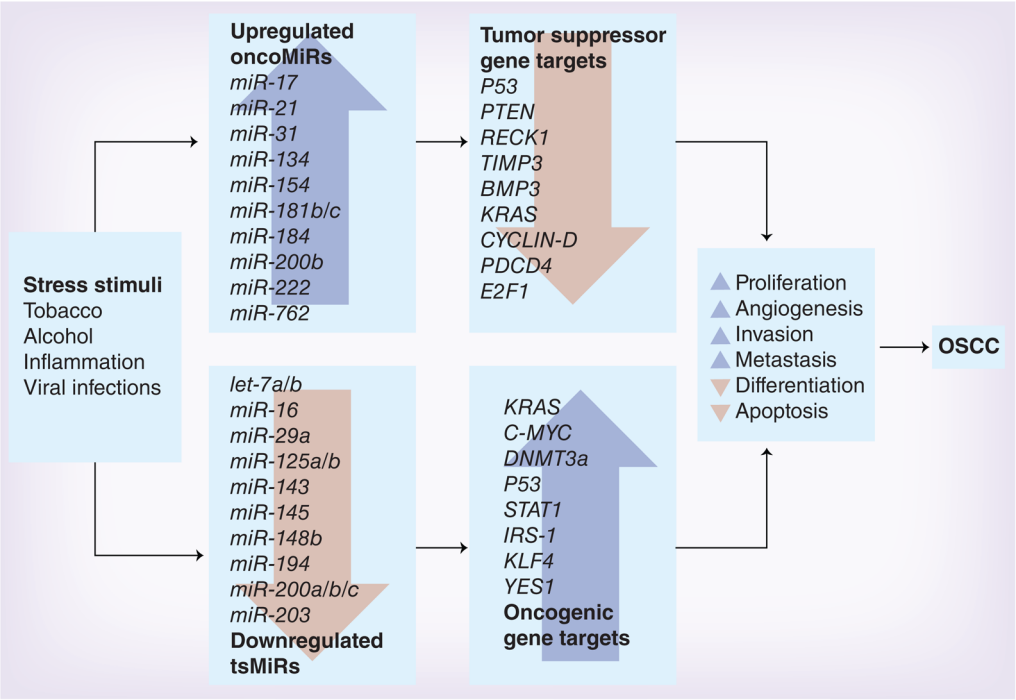

Figure 2. Role of miRNAs in the development and progression of oral squamous cell carcinoma

Two important epigenetic mechanisms support OSCC tumorigenesis: overexpression of oncogenic miRNAs (or oncoMiRs) and underexpression of tumor suppressor miRNAs (or tsMiRs). Listed in this figure are some of the most important miRNAs and their gene targets that have emerged as key regulators of gene expression in oral cancer.

OSCC: Oral squamous cell carcinoma.

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Table 1

Key genes frequently hypermethylated in oral cancer[†].

Gene	Locus	Function	Methylation frequency (%), n/N			Ref.
			Normal	*Adjacent tumor tissue*	*OSCC*	
APC	5q21	WNT signaling		6 (347)	13 (6/47)	[6]
BIRC5/SURVIVIN	17q25	Cell cycle progression			0	[9]
CACNA1G	17q22	Voltage-gated ion channel activity	0 (0/2)		0 (0/96)	[107]
CCNA1/CYCLIN A	13q12.3-q13	Cell cycle regulation		0 (0/9)	53 (42/78)	[17]
CDH1/E-CADHERIN	16q22.1	Cell adhesion		13 (6/47)	42 (20/47)	[6]
CHFR	12q24.33	Cell cycle regulation	0 (0/18)	8 (1/13)	46 (6/13)	[108]
DAPK	9q34.1	Apoptosis	0 (0/20)	60 (36/60)	68 (41/60)	[109]
DCC	18q21.1	Transcription coactivator	0 (0/30)	10 (5/48)	59 (54/92)	[36]
EDNRB	13q22	Receptor activity	0 (0/30)	10 (5/48)	73 (67/92)	[36]
EPCAM	2p21	Calcium-independent cell adhesion molecule			51 (37/72)	[110]
FHIT	3p14.2	Cell cycle progression		0 (0/13)	28 (8/29)	[111]
GSTP1	11q13	Glutathione transferase activity	0 (0/20)	0 (0/60)	0 (0/60)	[109]
KIF1A	2q37.3	Axon transport	0 (0/30)	10 (5/48)	72 (66/92)	[36]
MGMT	10q26.3	DNA damage response	0 (0/20)	27 (16/60)	52 (31/60)	[109]
MINT1	9q13-q21	Movement, exocytosis and adhesion	0 (0/2)		23 (22/96)	[107]
MINT2	15q11-q12	Protein binding and exocytosis	0 (0/2)		8 (8/96)	[107]
MINT27	1p36	Transcriptional regulator	0 (0/2)		16 (15/96)	[107]
MINT31	1p36	Transcriptional regulator	0 (0/2)		15 (14/96)	[107]
MLH1	3p21.3	DNA damage response	0 (0/200)		76 (38/50)	[25]
MSH2	2p21	DNA damage response and mismatch repair			50 (14/28)	[24]
Notch1	9q34.3	Transcription factor and receptor activity			6 (2/34)	[112]
$p14^{ARF}$	9p21	Cell cycle regulation	0 (0/2)		14 (13/96)	[107]
$p15^{INK4B}$/*CDKN2B*	9p21	Cell cycle regulation	0 (0/2)	0 (0/25)	23 (12/51)	[26]
$p16^{INK4A}$/*CDKN2A*	9p21	Cell cycle regulation	0 (0/20)	50 (30/60)	67 (40/60)	[109]
p53	17p13.1	Cell cycle regulation			4 (2/48)	[30]

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Gene	Locus	Function	Methylation frequency (%), n/N			Ref.
			Normal	*Adjacent tumor tissue*	*OSCC*	
RARβ	3p24.2	Transcription factor and receptor activity		62 (13/21)	73 (58/80)	[17]
RASSF1	3p21.3	Cell cycle progression		13 (6/47)	38 (18/47)	[6]
RASSF2	20p13	Cell cycle progression			28 (134/482)	[44]
RECK	9p13.3	Protein binding and peptidase inhibitor activity	0 (0/12)	30 (6/20)	55 (11/20)	[113]
RUNX3	1p36	WNT signaling	0 (0/10)	53 (16/30)	70 (21/30)	[114]
SFRP1	8p11.21	Protein binding			24 (10/42)	[115]
SFRP2	4q31.3	Protein binding			36 (16/44)	[115]
SFRP5	10q24	Protein binding			16 (7/43)	[115]
VHL	3p25.3	Transcription factor binding			0 (0/48)	[30]
WIF1	12q14.3	WNT signaling		9 (4/47)	42 (20/47)	[6]

†For a more comprehensive list, please refer to Supplementary Table 1.

OSCC: Oral squamous cell carcinoma.

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Table 2

Classification of histone deacetylase inhibitors.

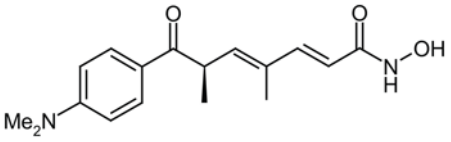
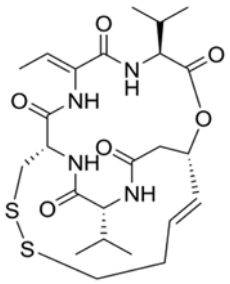
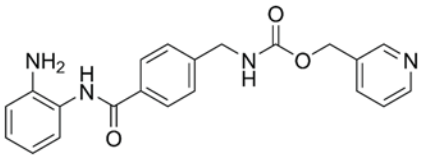
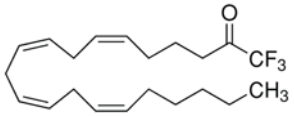
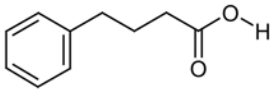
Histone deacetylase inhibitor group	Representative example	Chemical structure
Hydroxamic acids	Trichostatin A, Suberoylanilidehydroxamic acid	
Cyclic tetrapeptides and depsipeptides	FR901228	
Benzamides	MS-275	
Electrophilic ketones	Trifluoromethyl ketone	
Aliphatic acid compounds	Phenylbutyric acid	

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Table 3

Important miRNAs frequently altered in oral cancer[†].

miRNA	Regulation	Malignancy	Sample type	Method	Ref.
let-7a/7b	Down	OSCC	Cell lines	qRT-PCR	[102]
miR-16	Down	OSCC	Hamster cheek pouch	Microarray, qRT-PCR	[98]
miR-17-5p	Up	TSCC	Tissue	qRT-PCR (TaqMan® miRNA assay, 156miRNAs)	[91]
miR-21	Up	Leukoplakia	Tissue	TaqMan® low density arrays, qRT-PCR	[96]
miR-24	Up	TSCC	Tissue	qRT-PCR	[93]
miR-26a/b	Down	OSCC	Hamster cheek pouch	Microarray, qRT-PCR	[91,98]
miR-29a	Down	OSCC	Hamster cheek pouch	Microarray, qRT-PCR	[98]
miR-30a-3p	Up	TSCC	Tissue	qRT-PCR (TaqMan® miRNA assay, 156miRNAs)	[91]
miR-31	Up	OSCC	Plasma	qRT-PCR	[103]
miR-34b/c	Up	TSCC	Tissue	qRT-PCR (TaqMan® miRNA assay, 156miRNAs)	[91]
miR-99a	Down	TSCC	Tissue	qRT-PCR (TaqMan® miRNA assay, 156miRNAs)	[91]
miR-104	Up	TSCC	Tissue	qRT-PCR (TaqMan® miRNA assay, 156miRNAs)	[91]
miR-107	Down	TSCC	Tissue	qRT-PCR (TaqMan® miRNA assay, 156miRNAs)	[91]
miR-124a	Down	OSCC	Hamster cheek pouch	Microarray, qRT-PCR	[98]
miR-124b	Up	TSCC	Tissue	qRT-PCR (TaqMan® miRNA assay, 156miRNAs)	[91]
miR-125a/b	Down	OSCC	Saliva	qRT-PCR	[98,105]
miR-128a	Up	TSCC	Tissue	qRT-PCR (TaqMan® miRNA assay, 156miRNAs)	[91]
miR-132	Up	TSCC	Tissue	qRT-PCR (TaqMan® miRNA assay, 156miRNAs)	[91]
miR-133a/b	Down	TSCC	Tissue	qRT-PCR (TaqMan® miRNA assay, 156miRNAs)	[91]
miR-134	Up	TSCC	Tissue	qRT-PCR (TaqMan® miRNA assay, 156miRNAs)	[91]
miR-137	Up	TSCC	Tissue	qRT-PCR (TaqMan® miRNA assay, 156miRNAs)	[91]
miR-143	Down	OSCC	Hamster cheek pouch	Microarray, qRT-PCR	[98]
miR-145	Down	OSCC	Hamster cheek pouch	Microarray, qRT-PCR	[98]
miR-148b	Down	OSCC	Hamster cheek pouch	Microarray, qRT-PCR	[98]
miR-149	Down	TSCC	Tissue	qRT-PCR (TaqMan® miRNA assay, 156miRNAs)	[91]
miR-155	Down	OSCC	Hamster cheek pouch	Microarray, qRT-PCR	[98]

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miRNA	Regulation	Malignancy	Sample type	Method	Ref.
miR-181b	Up	Leukoplakia	Tissue	TaqMan® low density arrays, qRT-PCR	[96]
miR-181c	Up	TSCC	Tissue	qRT-PCR (TaqMan® miRNA assay, 156miRNAs)	[91]
miR-194	Down	TSCC	Tissue	qRT-PCR (TaqMan® miRNA assay, 156miRNAs)	[91]
miR-195	Down	TSCC	Tissue	qRT-PCR (TaqMan® miRNA assay, 156miRNAs)	[91]
miR-198	Up	TSCC	Tissue	qRT-PCR (TaqMan® miRNA assay, 156miRNAs)	[91]
miR-200a	Down	OSCC	Saliva	qRT-PCR	[105]
miR-200b	Up	OSCC	Hamster cheek pouch	Microarray, qRT-PCR	[98]
miR-203	Down	OSCC	Hamster cheek pouch	Microarray, qRT-PCR	[98]
miR-219	Down	TSCC	Tissue	qRT-PCR (TaqMan® miRNA assay, 156miRNAs)	[91]
miR-221	Up	OSCC	Hamster cheek pouch	Microarray, qRT-PCR	[98]
miR-345	Up	Leukoplakia	Tissue	TaqMan® low density arrays, qRT-PCR	[96]
miR-375	Down	OSCC	Tissue	qRT-PCR (TaqMan® miRNA assay)	[106]
miR-762	Up	OSCC	Hamster cheek pouch	Microarray, qRT-PCR	[98]

† For a more comprehensive list, please refer to Supplementary Table 2.

OSCC: Oral squamous cell carcinoma; qRT-PCR: Quantitative reverse transcriptase PCR; TSCC: Tongue squamous cell carcinoma.

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