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**Histone modifications: Targeting head and neck cancer stem cells**

Le J *et al.* Epigenetics and cancer stem cells

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**Abstract**

Head and Neck Squamous Cell Carcinoma (HNSCC) is the sixth most common cancer worldwide, and it is responsible for a quarter of a million deaths annually. The survival rate for HNSCC patients is poor, showing only minor improvement in the last three decades. Despite new surgical techniques and chemotherapy protocols, tumor resistance to chemotherapy remains a significant challenge for HNSCC patients. Numerous mechanisms underlie chemoresistance, including genetic and epigenetic alterations in cancer cells that may be acquired during treatment and activation of mitogenic signaling pathways, such as nuclear factor kappa-light-chain-enhancer-of activated B cell, that cause reduced apoptosis. In addition to dysfunctional molecular signaling, emerging evidence reveals involvement of cancer stem cells (CSCs) in tumor development and in tumor resistance to chemotherapy and radiotherapy. These observations have sparked interest in understanding the mechanisms involved in the control of CSC function and fate. Post-translational modifications of histones dynamically influence gene expression independent of alterations to the DNA sequence. Recent findings from our group have shown that pharmacological induction of post-translational modifications of tumor histones dynamically modulates CSC plasticity. These findings suggest that a better understanding of the biology of CSCs in response to epigenetic switches and pharmacological inhibitors of histone function may directly translate to the development of a mechanism-based strategy to disrupt CSCs. In this review, we present and discuss current knowledge on epigenetic modifications of HNSCC and CSC response to DNA methylation and histone modifications. In addition, we discuss chromatin modifications and their role in tumor resistance to therapy.

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**Key words:** Head and neck squamous cell carcinoma; Chromatin remodeling; Histone deacteylases inhibitor; Histone acetylation; Cancer initiating cell; Epigenetic target; Epigenetic marker; Oral squamous cell carcinoma; Tumor resistance

**Core tip:** Stem cells are long-lived, therefore their genome is subject to more stress from genetic mutations and epigenetic factors than their short-lived, differentiated progeny. Recent evidence strongly indicates that a subpopulation of tumor initiating cells, termed “cancer stem cells”, play a fundamental role in tumor heterogeneity, growth, and preservation. Cancer stem cells behavior is influenced by epigenetic events comprised primarily of DNA methylation and histone modifications that dynamically regulate gene expression and silencing.

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**INTRODUCTION**

There are approximately 560000 cases of head and neck cancer diagnosed worldwide each year and approximately 300000 deaths annually. This cancer type occurs in the head and neck region, involves the nasal and oral cavity, pharynx, and larynx and primarily occurs as squamous cell carcinoma (HNSCC)[[1-4](#_ENREF_1)]. Although HNSCC has well recognized risk factors, including tobacco use, excess alcohol consumption, and infection by high risk papillomaviruses[[5](#_ENREF_5),[6](#_ENREF_6)], we do not fully understand the mechanisms underlying its malignant progression[[5](#_ENREF_5)]. Our understanding of the molecular biology of HNSCC has significantly improved in the last few decades, contributing to the development of novel therapies targeted against pro-survival signaling circuitries, including the epidermal growth factor receptor (EGFR), vascular endothelial growth factor, receptor tyrosine kinases, interleukins, and phosphoinositide 3-kinase (PI3K) pathways, among others. Unfortunately, the long-term survival rate for HNSCC patients, which is 50% at five years after diagnosis, has remained consistent over the past thirty years[[3](#_ENREF_3),[7-9](#_ENREF_7)]. The incidence of HNSCC is much higher in developing nations, where it is the third most common malignancy in Asian countries compared to the sixth most common malignancy in Western countries[[10-12](#_ENREF_10)]. This discrepancy in incidence of HNSCC is associated with varying risk factors, such as chewing Betel quid in the Asia-Pacific region compared to consumption of tobacco and alcohol and/or human papillomavirus infection outside Asia[[1](#_ENREF_1),[13-17](#_ENREF_13)].

The poor long-term survival rates in HNSCC patients may be due to diagnosis of disease at an advanced-stage and development of chemoresistance[[8](#_ENREF_8),[18](#_ENREF_18)]. Numerous mechanisms underlie chemoresistance, including genetic and epigenetic alterations in cancer cells that may be acquired during treatment[[19](#_ENREF_19),[20](#_ENREF_20)] and the activation of mitogenic signaling pathways, such as nuclear factor kappa-light-chain-enhancer-of activated B cell (NFκB), that result in reduced apoptosis[[21](#_ENREF_21)]. Furthermore, the recurrence of cancers depend on a subpopulation of cancer stem cells (CSCs) that possess the unique and exclusive ability to self-renew and differentiate into nontumorigenic heterogenous cell types that maintain the tumor[[7](#_ENREF_7),[22-24](#_ENREF_22)]. Therefore, many factors play a critical role in the maintenance of tumor heterogeneity and CSC behavior, including the tumor microenvironment, genomic instability and the effect of genetic mutations and epigenetic changes on gene expression[[22](#_ENREF_22),[25-27](#_ENREF_25)].

In a significant number of HNSCC, tumor progression results from mutations in genes, such as *TP53*, *CDKN2A*, *HRAS*, *PTEN*, and *PIK3CA*. This causes alterations in cell signaling cascades (*e.g.,* PI3K/mTOR, NFκB, ERK, p53), resulting in aberrant cell growth, migration, and survival[[3](#_ENREF_3),[8](#_ENREF_8),[23](#_ENREF_23),[28](#_ENREF_28),[29](#_ENREF_29)]. Epigenetic changes also play a key role in regulating gene expression through histone modifications, DNA methylation, miRNA silencing and DNA repair mechanisms [HMT(Histone methyltransferases), HAT(Histone acetyltransferases), HDAC(Histone deacteylases) ncRNA, and lncRNA][[30-33](#_ENREF_30)]. Consequently, by identifying the molecular mechanisms that drive progression and recurrence of HNSCC, novel cancer therapeutics can be developed that improve the effectiveness of treatment and the rate of long-term survival in patients. In this review, we highlight the current understanding on cancer stem cells and the effects of epigenetic modifications on tumor behavior. We also discuss the latest findings on pharmacological manipulation of epigenetic circuitries that may result in the development of novel therapeutic strategies that target cancer stem cells.

**CANCER STEM CELLS**

Because normal stem cells are long-lived, their genome is subject to more stress from genetic mutations and epigenetic factors than their short-lived, differentiated progeny. The majority of oncogenic mutations in stem cells perturb central cellular processes that regulate cellular division, DNA damage repair, and signal transduction pathways[[24](#_ENREF_24),[25](#_ENREF_25),[34](#_ENREF_34)]. Certain HNSCC-related phenotypes that arise from mutations in oncogenes and tumor suppressor genes, such as *PIK3CA*, *TP63*, *PTEN*, *EGFR*, and *MET*, result in limitless replication potential, insensitivity to apoptotic signals, angiogenesis, invasion and metastasis[[28](#_ENREF_28),[35-38](#_ENREF_35)]. Therefore, tumors arise when stem cells lose their ability to regulate and maintain tissue form and function and when they show reduced control over apoptosis, cellular senescence and cellular proliferation. Additionally, although tumors are a population of malfunctioning cells, they are commonly characterized by histological features that resemble normal tissue[[39](#_ENREF_39)]. Similarly, hematopoietic cancers are comprised of identical neoplastic cells, but solid tumors from HNSCC consist of non-identical cells, resulting in phenotypic heterogeneity[[25](#_ENREF_25),[27](#_ENREF_27),[40-42](#_ENREF_40)]. Within the polyclonal tumor, there is a cellular hierarchy in which a small subpopulation of neoplastic cells with the highest potential for tumorigenesis and self-renewal are positioned at the top. The remaining bulk of the tumor primarily consists of well-differentiated non-tumorigenic cells that are susceptible to chemotherapy and radiation[[43-45](#_ENREF_43)]. In addition to HNSCC, solid tumors of the breast, brain, colon, lung and prostate also demonstrate a diverse array of cellular heterogeneity that increases genomic instability and adaptability of the tumor to its microenvironment[[25](#_ENREF_25),[46](#_ENREF_46),[47](#_ENREF_47)]. Recent evidence strongly indicates that a subpopulation of tumor initiating cells, termed ‘cancer stem cells’, play a fundamental role in tumor heterogeneity, growth, and preservation[[25](#_ENREF_25),[44](#_ENREF_44),[48](#_ENREF_48),[49](#_ENREF_49)]. The cancer stem cell hypothesis, first conceptualized by Bonnet and Dick[[44](#_ENREF_44)] in 1997, established that a subpopulation of human leukemic cells, positive for CD34 and negative for CD38 cell surface markers, initiates human acute myeloid leukemia in Non-obese diabetic/Severe combined immunodeficient (NOD/SCID) mice. The following observations support the cancer stem cell hypothesis: (1) only a subpopulation of tumor cells within a tumor mass grow in immunodeficient mice; (2) the subpopulation of tumor cells generate both CSCs and heterogeneous nontumorigenic cancer cells; and (3) cancer stem cells self-renew, as revealed by serial transplantation assays[[22](#_ENREF_22),[44](#_ENREF_44),[50](#_ENREF_50)]. The frequency of CSCs is relatively low in HNSCC, lung squamous cell carcinoma, lung adenocarcinoma, and human pancreatic adenocarcinoma, but xenotransplantation assays greatly increase their frequency[[51](#_ENREF_51)].

***Cancer Stem Cell surface markers***

CSCs were first discovered in solid tumors in 2003[[52](#_ENREF_52)], and the isolation of CSCs in HNSCC, based on the CD44+ cell surface marker, occurred in 2007[[18](#_ENREF_18)]. In that study, approximately 70% of NOD/SCID mice receiving CD44+ tumor cell xenografts showed tumor formation compared to 1% of mice receiving CD44- xenografts. In addition to their association with CSCs in HNSCC[[53-56](#_ENREF_53)], CD44+ cells also play a role in chemoresistance. Genes associated with chemoresistance, including ABCB1, ABCG2, CYP2C8 and TERT, are upregulated in CD44+ cells compared to CD44- cells[[57](#_ENREF_57)]. Furthermore, CD44+ HNSCC cells express high levels of blymphoma Mo-MLV insertion region 1 homolog (Bmi-1), a self-renewal and oncogenic protein associated with poor survival and tumor aggressiveness[[18](#_ENREF_18),[58-62](#_ENREF_58)]. Different isoforms of CD44 differentially modify the behavior of HNSCC. For instance, the v3, v6, and v10 isoforms of CD44 promote HNSCC tumor migration, invasion, and metastasis[[63](#_ENREF_63),[64](#_ENREF_64)] and confer chemoresistance in other solid tumors, attributes commonly associated with the chemo- and radio-resistant fractions of cancer stem cells[[65](#_ENREF_65)]. Therefore, CD44 is used to identify CSCs, and it promotes many of the biological characteristics associated with cancer “stemness”. These characteristics include the tumor growing in a sphere in suspension, unrestricted cellular proliferation, enhanced migration, tumor invasion, and resistance to chemotherapy and ionizing radiation therapy. CD24 and CD133 (also known as Prominin 1) are also CSC cell surface markers[[66-68](#_ENREF_66)].

The increased enzymatic activity of aldehyde dehydrogenase 1 (ALDH1) is commonly used to identify normal pluripotent cells and tumor cells harboring “stemness” potential in various solid tumors, including HNSCC[[51](#_ENREF_51),[69-75](#_ENREF_69)]. ALDH is a detoxifying enzyme involved in the oxidation of intracellular aldehydes and was initially described for its role in hematopoietic stem cell self-renewal via reduction of retinoic acid activity[[76](#_ENREF_76),[77](#_ENREF_77)]. The presence of ALDH1-positive tumor cells correlates with poor clinical outcome in breast cancer[[69](#_ENREF_69)], ovarian cancer[[78](#_ENREF_78)], papillary thyroid carcinoma[[79](#_ENREF_79)], and pancreatic adenocarcinoma[[80](#_ENREF_80)], among other solid tumors[[70](#_ENREF_70),[81-83](#_ENREF_81)].

It is believed that HNSCC progression and invasion, in addition to resistance to non-surgical therapies, may be regulated by the rare population of CSCs[[18](#_ENREF_18),[43](#_ENREF_43),[84](#_ENREF_84),[85](#_ENREF_85)]. Therefore, to effectively treat this type of cancer, we must develop a therapy that can target and eliminate CSCs.

**EPIGENETICS OF HEAD AND NECK CANCER AND ITS STEM CELLS**

***Basic concepts of epigenetic regulation***

**DNA methylation:** When exploring the molecular mechanisms underlying cancer, DNA methylation is the most commonly studied epigenetic alteration[[86-88](#_ENREF_86)]. DNA methylation patterns occur in early and precancerous stages and most frequently discovered in tumors compared to normal tissues[[89](#_ENREF_89),[90](#_ENREF_90)]. Methylation occurs sporadically and is globally distributed in mammals throughout the genome at cytosine-phospho-guanine (CpG) dinucleotide sequences, as revealed by immunofluorescent labeled 5-methylcytosine. Without considering CpG-rich islands (approximately 1 kilobase in length), there is a low, but global level of methylation in specific CpG sequences throughout the entire mammalian genome[[26](#_ENREF_26),[91](#_ENREF_91)]. Therefore, aberrant DNA methylation of these CpG islands or specific sequences can lead to oncogenic activation via silencing of tumor suppressor gene expression[[92](#_ENREF_92),[93](#_ENREF_93)]. Hypomethylation is associated with activation of oncogenes, and hypermethylation is associated with the silencing of tumor suppressor genes. Both mechanisms induce genomic instability and play a dominant role in tumor initiation and progression[[90](#_ENREF_90),[94](#_ENREF_94)]. The most common types of DNA methylation in tumors are hypermethylation of CpG islands and global hypomethylation[[89](#_ENREF_89)]. Hypermethylated CpG islands are often associated with gene promoters; thus, methylation results in a transcriptionally inactive gene. In contrast, methylation of DNA sequences further from promoter sequences has less of an effect on transcription[[26](#_ENREF_26)].

**Histone modifications**: In addition to DNA methylation, the chromatin architecture can be remodeled by a network of protein mediators called histones that play an important role in gene regulation by compacting DNA. Histones can be post-translationally modified at the amino-terminal ends by acetylation, methylation, phosphorylation, sumoylation, ubiquitination, and ADP-ribosylation[[95](#_ENREF_95)]. These modifications result in gene transcription through the uncoiling of chromatin or gene silencing through compacting DNA[[96](#_ENREF_96)]. HAT, HMT, and HDAC are key co-factors that modify histones and produce the epigenetic changes observed in cancer. Histone acetylation, deacetylation and methylation are the major marks associated with transcriptional activity. Histone acetylation results in chromatin decondensation, promotion of transcription, and inhibition of DNA methylation, and is often correlated with formation of euchromatin. In contrast, histone deacetylation is the predominant epigenetic influence in transcriptional gene silencing[[95](#_ENREF_95),[97](#_ENREF_97),[98](#_ENREF_98)]. In general, histone modifications modulate a diverse array of biological processes, including gene regulation, DNA repair, mitosis and meiosis via chromosome remodeling[[99](#_ENREF_99)].

**Histone acetylation and deacetylation:** Dysregulation of the exquisite interplay between acetylation and deacetylation controlled by HAT and HDAC is coupled to the initiation and progression of cancer, cellular plasticity, inflammation, and dynamic transformation in metabolic cascades[[100](#_ENREF_100),[101](#_ENREF_101)]. In addition to the histone substrate peptides described in[[102](#_ENREF_102)], HAT is associated with non-histone proteins, transcription co-factors, such as p53, p65, c-MYC, NFκB, STAT3 (signal transducer and activator of transcription 3) and BRCA1 (breast cancer 1), among others[[30](#_ENREF_30),[103](#_ENREF_103)]. In particular, acetylation of the p53 tumor suppressor and pro-apoptotic protein by the CBP (CREB-binding protein)/p300 family of HATs has been extensively reviewed in[[104](#_ENREF_104),[105](#_ENREF_105)]. Modification of p53 is associated with increased DNA binding affinity, transcriptional activity[[106](#_ENREF_106),[107](#_ENREF_107)] and protein stability[[108](#_ENREF_108)]. Similar to p53, CBP/p300 is associated with the pro-proliferative and oncoproteins previously listed, and its expression impacts a variety of human diseases, such as leukemia[[109](#_ENREF_109),[110](#_ENREF_110)], lung cancer[[111](#_ENREF_111)], colon cancer[[112](#_ENREF_112)], bladder cancer[[113](#_ENREF_113)] and prostate cancer[[114-116](#_ENREF_114)]. CBP/p300 is also associated with transcription factors involved in heart disease[[117](#_ENREF_117),[118](#_ENREF_118)], diabetes[[119](#_ENREF_119),[120](#_ENREF_120)] and neurological disorders[[121](#_ENREF_121),[122](#_ENREF_122)].

**Histone methylation:** Histone methylation is the third major epigenetic process that affects transcriptional activation via chromatin remodeling. Similar to previously described post-translational histone modifications, methylation and demethylation of amino acids at different sites on histones either promotes or prevents transcriptional activity[[123](#_ENREF_123)]. For example, methylation of lysine residues is associated with transcription and DNA repair, but methylation of arginine residues is only associated with transcription [[95](#_ENREF_95),[124](#_ENREF_124),[125](#_ENREF_125)]. Histone H3 is methylated at different lysine sites, including K4, K9, K27, K36, and K79, that experience various methylated states, including monomethylated, dimethylated, and trimethylated. Therefore, the epigenetic modification of the chromatin depends on the location and state of methylation[[126](#_ENREF_126),[127](#_ENREF_127)]. K9 and K27 methylation is associated with heterochromatin formation and inactive transcription. In contrast, K4 methylation is associated with euchromatin formation and active transcription[[128](#_ENREF_128),[129](#_ENREF_129)].

**HAT and HDAC inhibitors:** The development of HAT inhibitors (HATi) are in the early stages of preclinical studies. Although drugs that regulate HDAC activity are being used for cancer treatment, there is great interest in developing HAT inhibitors as a potential treatment for cancer and other human diseases[[130](#_ENREF_130)]. Several natural compounds effectively inhibit HAT activity. For example, Marcu *et al*[[131](#_ENREF_131)] demonstrated that curcumin inhibits HAT activity by promoting proteasome-dependent degradation of CBP/p300 in both prostate cancer cells and in HDAC inhibitor-induced peripheral blood lymphocytes. In addition, epigallocatechin-3-gallate and plumbagin are selective inhibitors of CBP/p300[[132-134](#_ENREF_132)]. The potential for HDAC inhibitors (HDACi) to serve as cancer chemotherapeutics has been examined in clinical trials due to the role of HDAC in genome stability, proliferation, differentiation, apoptosis, and metabolism. A current list of HDACi under clinical investigation can be found in a review by Li and Zhu[[135](#_ENREF_135)] that focuses on HDAC and its clinical implications in cancer therapy.

In summary, epigenetic modifications constitute the next frontier in tumor biology research. Post-translational modification of histones dynamically influences gene expression independent of alterations to the DNA sequence. These mechanisms are often mediated by histone linkers, proteins associated with the recruitment of DNA-binding proteins, HDAC I and II interacting proteins and transcriptional activators, coactivators or corepressors. Therefore, histones are molecular markers of epigenetic changes[[136](#_ENREF_136)].

***Epigenetic regulation of HNSCC***

In HNSCC and other carcinomas, the combination of genetic and epigenetic factors affect gene expression, resulting in altered downstream cellular signaling pathways that regulate tumor growth, anti-apoptosis, DNA repair, resistance to extrinsic factors, angiogenesis, and epithelial-mesenchymal transition (EMT)[[31](#_ENREF_31),[137-140](#_ENREF_137)]. Although both genetics and epigenetics may affect the initiation and progression of HNSCC, epigenetic factors regulate gene expression in the absence of genomic mutations[[19](#_ENREF_19),[141](#_ENREF_141),[142](#_ENREF_142)]. Therefore, epigenetics is defined as a stable heritable phenotype passed on through either mitosis or meiosis, resulting in changes in chromosome characteristics without inducing genome alterations, as proposed by Conrad Waddington in the early 1940s[[143-145](#_ENREF_143)].

Tumor development is a multi-stage process that requires the accumulation of numerous genetic mutations and often results in gain-of-function in oncogenes and loss-of-function in tumor suppressor genes[[146-150](#_ENREF_146)]. In addition to genetic mutations, tumor development and progression is extensively influenced by changes in gene expression independent of alterations in the DNA sequence, a mechanism known as epigenetic modification. Epigenetic events are comprised primarily of DNA methylation and histone modifications that dynamically regulate gene expression and silencing[[19](#_ENREF_19),[31](#_ENREF_31),[141](#_ENREF_141),[142](#_ENREF_142),[151](#_ENREF_151)]. These dynamic processes occur within the chromatin that is packed into the nucleus through interactions with core histone proteins.

The effect of chromatin on cellular behavior depends on how tightly DNA is spooled around H2A, H2B, H3 and H4 core histones[[152](#_ENREF_152)]. Together, histones and DNA form nucleosomes, the fundamental units of chromatin. Gene expression is driven by the ability of chromatin to fold and unfold in a process that requires rapid acetylation/deacetylation of the histone core, resulting in alterations in the cellular response to environmental cues[[153](#_ENREF_153)].

**DNA methylation in HNSCC:** In Demokan and Dalay’s extensive review[[89](#_ENREF_89)] of DNA methylation in head and neck cancers, they provide a list of the most frequently methylated genes. In this list, the hypermethylated genes include the following: (1) Adenomatous polyposis coli, which is the most common gene methylated in HNSCC[[154](#_ENREF_154),[155](#_ENREF_155)]; (2) p16, a cell cycle controller encoded by the *CDKN2A* gene, which plays a critical role in inducing cellular senescence in tumor cells and is downregulated via promoter hypermethylation[[156-167](#_ENREF_156)]; and (3) p14, also known as ARF, that in combination with p16 is involved in regulating the cell cycle and in activating the p53 tumor suppressor gene by inhibiting MDM2[[168](#_ENREF_168)]. Surprisingly, in 96 human samples of oral squamous cell carcinoma, methylation of p14ARF is associated with a good prognosis, methylation of MINT1 and MINT31 is associated with poor prognosis, and DCC methylation is associated with increased bone invasion by squamous cell carcinoma from the gingiva[[169](#_ENREF_169)]. Notably, Ogi *et al*[[159](#_ENREF_159),[169](#_ENREF_169)] also identified methylated MINT31 as an independent predictor of outcome and showed its association with the T4 disease group, according to the Union for International Cancer Control classification. *RASSF1A* is a tumor suppressor gene that is frequently lost in tumors, including HNSCC. *RASSF1A* is involved in the maintenance of genomic stability and is highly mutated in poorly differentiated HNSCC compared to moderate and well-differentiated HNSCC[[154](#_ENREF_154), [159](#_ENREF_159),[160](#_ENREF_160),[163](#_ENREF_163),[165](#_ENREF_165),[167](#_ENREF_167),[170](#_ENREF_170),[171](#_ENREF_171)]. RASSF2 is a novel Ras-associated protein that negatively regulates Ras signaling[[172](#_ENREF_172)]. RASSF2 binds directly to K-Ras in a GTP-dependent manner promoting apoptosis and cell cycle arrest; however, RASSF2 weakly interacts with H-Ras. In solid tumors, including human colorectal cancer and HNSCC, RASSF2 is frequently silenced by DNA methylation at 5′ CpG islands[[167](#_ENREF_167),[173](#_ENREF_173)].

Other interesting genes methylated in head and neck cancer include EDNRB, a member of the G protein-coupled receptor family that encodes endothelin receptor type B protein; EDNRB is methylated in 97% of primary HNSCC tissues[[174](#_ENREF_174)]. EDNRB is involved in the development and function of blood vessels, cellular growth and mitosis[[174](#_ENREF_174)]. Another gene methylated in HNSCC is RARB, which encodes retinoic acid receptor beta and restricts cell growth by altering gene expression. Hypermethylation of RARB results in loss of function and reduced control of transcription[[154](#_ENREF_154),[162](#_ENREF_162),[163](#_ENREF_163),[167](#_ENREF_167),[175](#_ENREF_175),[176](#_ENREF_176)]. Currently, only a few methylated genes can predict the clinical outcome of HNSCC patients. It is unknown how methylated genes correlate with cancer therapy, patient response and tumor progression and behavior. Methylation analysis techniques have revealed that methylation patterns are not affected by external factors and are increased during cancer progression. Therefore, as with stem cell surface markers, increased sensitivity and specificity of quantitative methodologies for DNA methylation analyses will allow scientists to develop prognostic tools for clinical evaluation of head and neck cancer.

**Histone methylation in HNSCC:** Mancuso *et al*. showed that the level of H3K4 methylation is significantly different in normal mucosa compared to oral squamous cell carcinoma (OSCC) tissues, with dimethylated K4 increased and trimethylated K4 decreased. A similar trend was observed in oral leukoplakias compared to the pathological sample[[177](#_ENREF_177)]. H3K9 and H3K27 are targets for methylation by enhancer of zeste homolog 2 (EZH2), a member of the Polycomb-group family, resulting in gene silencing via chromatin condensation[[178-181](#_ENREF_178)]. Interestingly, overexpression of EZH2 is associated with malignancy and prognosis of a variety of cancers, including breast[[182](#_ENREF_182),[183](#_ENREF_183)], prostate[[184-186](#_ENREF_184)], gastric[[187](#_ENREF_187)], hepatic[[188](#_ENREF_188)], bladder[[189](#_ENREF_189),[190](#_ENREF_190)] and oral squamous cell carcinoma[[129](#_ENREF_129),[191](#_ENREF_191)]. Wei *et al* showed that increased expression of EZH2 is associated with dysplasia and malignant transformation. Similarly, Kidani *et al* revealed that overexpression of EZH2 is associated with tumor progression, malignancy and poor prognosis in OSCC. Collectively, these data reveal that different histone methylation patterns can greatly influence gene expression in cancer, thereby affecting malignant behavior.

**Histone acetylation in HNSCC:** Early evidence suggested that histones and their modifiers are involved in sophisticated processes that modulate tumor behavior and cellular phenotype. We recently reported that chromatin folding in HNSCC during tumor response to environmental cues dynamically modulates tumor behavior and cellular phenotype[[151](#_ENREF_151)]. We found that HNSCC cell lines are hypoacetylated compared to normal mucosa controls (Figure 1A). Furthermore, we found that endothelial cell-secreted factors, but not fibroblast cell-secreted factors, are able to trigger the acetylation of histones in tumor cells (also referred to as tumor histones) (Figure 1B). In fact, paracrine-induced histone modifications resulted in enhanced expression of Bmi-1, a transcriptional repressor upregulated in a variety of cancers and associated with tumor aggressiveness, and poor survival along with the expression of vimentin, a canonical marker of EMT (Figure 1B)[[192-199](#_ENREF_192)]. Similar to our *in vitro* findings, human HNSCC samples presented coexpression of acetylated histone 3 and vimentin in the proximity of normal endothelial cells (Figure 1C-white dashed line) next to the tumor invasion front in human HNSSC samples (Figure 1C- yellow dashed line). Therefore, acetylation of tumor histones are associated to changes in cellular behavior, phenotype and associated to increased invasion. In fact, malignant tumors derived from epithelial cells (carcinomas) are known to undergo EMT that precedes local invasion and metastasis of cancer cells[[200-204](#_ENREF_200)]. EMT is characterized by loss of cell adhesion, increased motility, aggressive behavior, acquisition of an elongated fibroblastoid morphology and expression of vimentin[[200](#_ENREF_200),[205](#_ENREF_205),[206](#_ENREF_206)], similar to what we observe with pharmacological inhibition of HDAC in HNSCC cell lines (Figure 2-HN6 and HN13 cells). Interestingly, cellular morphology is not altered and vimentin is not induced in normal epithelial cells (NOK-SI) treated with HDAC inhibitors, suggesting that hyperacetylation of chromatin differentially modulates normal and neoplastic cells (Figure 2). However, changes in the acetylation of HNSCC chromatin also triggered an unexpected phenotype, which was the loss of CSCs. HNSCC treated with Trichostatin A, a histone deacetylase inhibitor, lose the ability to generate and maintain tumor spheres and experience rapid reduction in the enzymatic activity of ALDH1 (Figure 3)[[151](#_ENREF_151)]. It has been suggested that epigenetic signals play a major role in stem cell control through deacetylation of histones, which promotes chromatin condensation and reactivation of stem cell-like transcription programs[[34](#_ENREF_34)]. These striking findings suggest that chromatin acetylation selectively disrupts the physiological requirements for maintenance of CSC. Indeed, chromatin acetylation has long been known to induce cellular differentiation and restrict cellular transformation of normal cells[[34](#_ENREF_34),[207](#_ENREF_207),[208](#_ENREF_208)].

In summary, histone modifications via methylation, acetylation and deacetylation play a critical role in transcriptional activation and gene expression. Aside from the physiological maintenance of cellular homeostasis, aberrant alterations in histone methylation proteins and/or an imbalance in the HAT/HDAC network results in dysfunctions in cellular processes, such as proliferation, differentiation, DNA repair and apoptosis. Importantly, post-translational histone modification and DNA methylation can have similar patterns in the same cancer type. For example, a study by Piyathilake *et al* revealed that patterns of global DNA and histone methylation are similar in different human mucosal tissues (*e.g.,* normal, dysplastic and squamous cell carcinoma). Using immunohistochemical analysis, they also found that global DNA methylation and H3 methylation at lysine 4 and lysine 9 are significantly higher in dysplastic lesions and carcinoma cells compared to normal oral epithelium[[209](#_ENREF_209)]. Therefore, when developing methods and techniques for identifying epigenetic markers in premalignant cells, we must consider analyzing both global DNA and histone methylation levels concurrently in the progression of cancer. In conclusion, the previously described epigenetic alterations are closely associated with tumorigenesis and malignancy in many types of cancers. As a result, genomic instability affects numerous intracellular signaling cascades. We will discuss the NFκB signaling pathway in the next section.

**TUMOR HISTONE MODIFICATIONS: EVIDENCE FOR AN EPIGENETIC MECHANISM RESPONSIBLE FOR ACQUIRED TUMOR RESISTANCE TO THERAPY**

NFκB is an epigenetic modifier that plays a major role in malignant transformation[[210](#_ENREF_210)], and this pathway serves as a target for epigenetic drugs[[211-213](#_ENREF_211)]. We, along with others, have previously reported that constitutive activation of NFκB signaling is often observed in HNSCC, suggesting a common epigenetic mechanism in HNSCC biology[[214](#_ENREF_214),[215](#_ENREF_215)]. Indeed, activation of NFκB signaling in HNSCC induced chromatin compaction and acquisition of resistance to chemotherapy[[216](#_ENREF_216)]. NFκB is active following translocation to the nucleus, a process that is regulated by the IκB kinase (IKK) complex. IκB proteins are targeted for degradation by phosphorylation, which permits nuclear translocation. Nuclear NFκB binds to target DNA sequences and modulates the expression of target genes involved in immune response, cell growth, and cell survival[[217](#_ENREF_217)]. Targeted inhibition of NFκB through IKKα and IKKβ silencing resulted in disrupted accumulation of nuclear phopho-p65, increased acetylation of histone 3 and accumulation of BRCA1. Collectively, we showed that NFκB epigenetically modulates chromatin organization and recruits BRCA1 to the nucleus. Indeed, histone 3 is acetylated following loss of NFκB, resulting in decondensation of tumor chromatin and sensitization of head and neck tumors to chemotherapy. This indicates that the effect of NFκB on chromatin organization directly influences tumor response to therapy. As proof of concept, administration of HDAC inhibitors recapitulate the effects of NFκB targeted inhibition by promoting chromatin decondensation and sensitizing tumor cells to chemotherapy, resulting in increased sensitivity of tumor cells to chemotherapy (Figure 4).

In addition to chemoresistance, activation of NFκB signaling increases the number of tumor spheres, indicating a broader role of NFκB as an epigenetic switch in CSCs. Notably, NFκB signaling is required for the development of tumor spheres in breast, cervical and head and neck cancers[[218](#_ENREF_218)] (Almeida and Castilho, submitted). We established that by controlling tumor histones we can dynamically regulate the behavior and number of HNSCC and its CSCs[[151](#_ENREF_151)]. Epigenetic signals may play a major role in stem cell control through deacetylation of histones, which promotes chromatin condensation and reactivation of stem cell-like transcription programs[[34](#_ENREF_34)]. Aligned with previous reports[[219-221](#_ENREF_219)], we showed that HNSCC tumor cell lines have a subpopulation of CSC, as detected by elevated ALDH activity, and clonogenic potential[[151](#_ENREF_151)]. This subpopulation of CSCs is highly tumorigenic and can self-renew, as observed by serial transplantation assays[[37](#_ENREF_37)]. By inhibiting HDAC and inducing acetylation of tumor histones, we found that CSCs lose their “stemness”, as evidenced by a reduction in ALDH+ cells and progressive disruption of tumor spheres. These findings indicate that HDAC inhibition disrupts the physiological requirements for CSC maintenance. Indeed, chromatin acetylation induces cellular differentiation and restricts cellular transformation[[207](#_ENREF_207),[208](#_ENREF_208)].

Altogether, HNSCC behavior appears dependent on dynamic changes in chromatin organization and subsequent gene transcription. Unlike stable DNA modifications mediated by methylation, acetylation of histones dynamically alters gene expression, thereby influencing tumor behavior following changes in the microenvironment as observed during administration of secreted factor from endothelial cells[[151](#_ENREF_151)] and expression of tumor aggressiveness markers[[222-225](#_ENREF_222)].

**CONCLUSION**

The role of epigenetic modifications in HNSCC warrants further investigation. Compared to histone modifications, the role of DNA methylation in regulating gene expression is better characterized. Nonetheless, recent studies have correlated the effects of histone acetylation in the dynamic process of tumor adaptation to its microenvironment and the acquisition of a resistant phenotype[[151](#_ENREF_151)]. The identification of the NFκB signaling pathway as an epigenetic modulator of tumor behavior and resistance to chemotherapy further improved our knowledge in the intricate molecular mechanism of HNSCC and further clarified our understanding of the NFκB signaling pathway[[216](#_ENREF_216)]. Novel therapeutic strategies can now be developed that target epigenetic alterations driven by histone modifications, and the NFκB signaling may serve as an ideal coadjuvant target for therapy. The development of personalized therapies specific for tumor subtypes, in this case tumors with active NFκB signaling, holds the promise of preventing tumor resistance and sensitizing tumors to chemotherapy. Recent advances in genome sequencing, including next-generation sequencing (NGS), have also improved our understanding of altered molecular signaling in HNSCC. NGS was used to identify single-base changes and larger structural variants characterized by insertions, deletions, translocations and viral insertions in HNSCC[[3](#_ENREF_3),[226](#_ENREF_226)]. Interestingly, NGS also revealed that HNSCC have a significant number of mutations in histones, histone modifiers, transcriptional activators and coactivators, and transcription regulators, further emphasizing the complexity of tumor signaling[[30](#_ENREF_30)]. Collectively, emerging knowledge about tumor behavior and how it correlates with dynamic changes in gene expression mediated by epigenetic events have substantially clarified the concept that successful therapeutic strategies will require targeting of genetic and epigenetic pathways.

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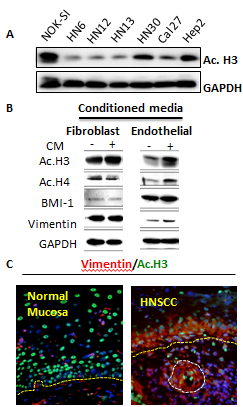
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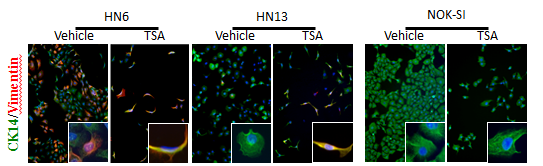
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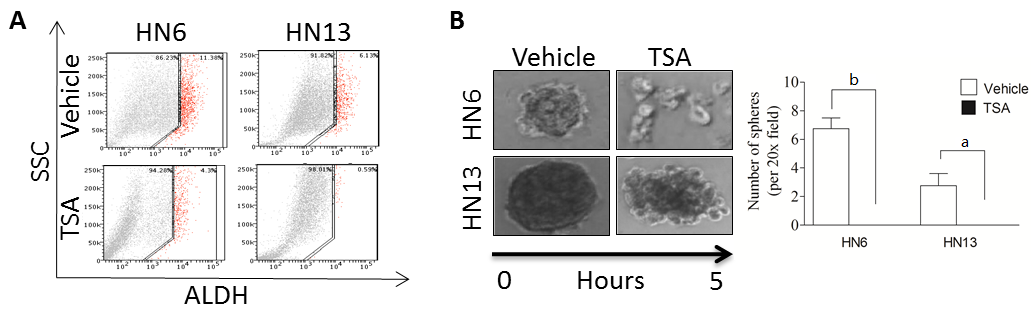
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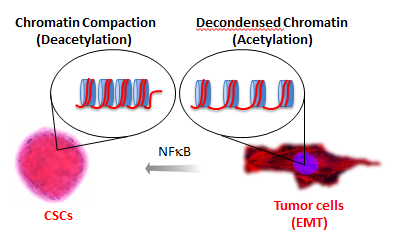
**Figure 1 Data represents acetylation status of histone 3 in Head and Neck Squamous Cell Carcinoma by Giudice *et al.*** A: Tumor cells present hypoacetylation of histone 3 (ac.H3) in a panel of Head and Neck Squamous Cell Carcinoma (HNSCC) compared to control cells (NOK-SI); B: Endothelial cell-secreted factors are capable of inducing ac.H3 while fibroblast cell-secreted factors cannot. Also, endothelial cell-secreted factors induce increased expression of BMI-1 and vimentin compared to the fibroblast counterpart; C: Representative examples of human samples of normal oral mucosa and HNSCC. Note acetylated tumor cells (Ac. H3-FITC) with high levels of the epithelial-mesenchymal transition marker vimentin (TRICT) are localized at the invasion front of HNSCC (arrow). Normal mucosa display acetylated cells distributed throughout the epidermis but do not express vimentin.ac. H3: Acetyl histone 3; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; CM: Conditioned Media; BMI-1: B lymphoma Mo-MLV insertion region 1 homolog; NOK-SI: Normal oral epithelial keratinocytes.



**Figure 2 Figure from Giudice *et al* depicting chemically-induced chromatin acetylation leading to activation of the epithelial-mesenchymal transition phenotype**. Inhibition of HDAC induces vimentin expression in HNSCC cells and EMT. Vehicle treated HNSCC cells (HN6 and HN13) present an epithelioid shape and express CK14. Administration of TSA result in acquisition of a fusiform morphology and expression of vimentin. Normal keratinocytes (NOK-SI) are not sensitive to EMT upon administration of TSA. TSA: Trichostatin A; CK14: Cytokeratin 14; EMT:Epithelial-mesenchymal transition; HDAC: Histone deacteylases; HNSCC: Head and Neck Squamous Cell Carcinoma; NOK-SI: Normal oral epithelial keratinocytes.



**Figure 3 Data from Giudice *et al* showing the impact of histone deacteylases inhibitor on the population of CICs.** A: Fluorescence-activated cell sorting of ALDH+ cells demonstrates that HNSCC cell lines have a high number CICs, and that administration of TSA reduced total number of ALDH+ cells; B: HDACi (TSA) disrupts tumor spheres as depicted in representative images of tumor spheres and by quantification of spheres (HN6 b*P* < 0.01, HN13 a*P* < 0.05).TSA: Trichostatin A; ALDH: Aldehyde dehydrogenase; SCC: Side scatter of light; HDACi: Histone deacteylases inhibitors; HNSCC: Head and Neck Squamous Cell Carcinoma.



**Figure 4 Data from Almeida *et al* proposing the mechanism for nuclear factor kappa-light-chain-enhancer of activated B cells driven resistance to chemotherapy in Head and Neck Squamous Cell Carcinoma.** Chromatin undergo normal compaction and decondensation through the acetylation of core histones organized in nucleosomes. Acetylation of tumor histones driven by expression of NFκBinfluences tumor behavior and plasticity of Cancer Stem Cells. NFκB: Nuclear factor kappa-light-chain-enhancer of activated B cells; EMT: Epithelial to mesenchymal transition.