Biologic Significance of 5-Hydroxymethylcytosine Expression in Oral Mucosal Epithelial Dysplasia and Oral Squamous Cell Carcinoma

Biological significance of 5-hydroxymehylcytosine expression in oral mucosal epithelial dysplasia and oral squamous cell carcinoma

A Thesis Presented by

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ABSTRACT

Current modeling postulates that the development of cancer is driven by the accumulation of genetic mutations and epigenetic modifications, resulting in a clonal population of cells with deregulated growth characteristics. Three epigenetic mechanisms have been well documented, including DNA methylation, histone modifications and non-coding RNAs. Of these, DNA methylation is the best studied epigenetic alteration in cancer. Characterization of the expression of the epigenetic marker 5-hydroxymethylcytosine (5-hmC) in human and experimental melanoma and its precursors has established it as an important functionally relevant epigenetic biomarker in cancer. We therefore posited that 5-hmC in oral premalignant lesions and oral squamous cell carcinomas represents an opportunity to identify a novel epigenetic molecular pathway that may serve as a biomarker for early diagnosis of oral neoplasms and subsequent improvements in the prognosis of oral cancer. A total of sixty-six histologic samples (N=66) were obtained from the Harvard School of Dental Medicine affiliated bio-archives. Immunohistochemistry for 5-hmC was performed on nine cases diagnosed as fibromas (F, n_F=9) with uninvolved margins used to evaluate non-inflamed/non-cancerous mucosa; nine cases diagnosed as frictional keratosis (FK, n_{FK}=9); ten cases diagnosed as lichen planus (LP, n_{LP}=10); fifteen cases diagnosed as moderate-to-severe oral epithelial dysplasia (OED, n_{OED}=15) and twenty-three cases diagnosed as oral squamous cell carcinoma (oSCC, n_{oSCC}=23). Human cell lines of oral keratinocytes from benign oral mucosa, oral epithelial dysplasia and four types of oral squamous cell carcinoma were also evaluated to detect the expression of 5-hmC genomic DNA. Finally, immunohistochemistry for 5-hmC was also performed in histologic samples of a murine model where oral squamous cell carcinoma was induced with the carcinogen 4-nitroquinoline-1-oxide (N=10). Five samples consisted of non-inflamed/non-neoplastic normal dorsal tongue mucosa (T, n_T=5) and five consisted of oral squamous cell carcinoma (mSCC, n_{mSCC}=5). Progressive loss from benign and reactive/inflammatory oral mucosal lesions to oral epithelial dysplasia and oral squamous cell carcinoma was observed in patient samples. Likewise, distinct patterns with regard to the distribution of staining was noted in benign non-inflamed lesions versus reactive inflammatory lesions such as frictional keratosis and lichen planus, providing further explanation germane for the understanding of the responses of the oral mucosa to tissue injury. The finding in human tissue of loss of 5-hmC immunoreactivity within dysplasia and carcinoma were substantiated in human cell lines at a DNA level, and further validated in an *in vivo* experimental model of murine oral SCC. In aggregate, our results confirm the potential importance of loss of 5-hmC as a novel epigenetic mark in oral SCC at both diagnostic and biological levels, and establish a murine model of oral SCC induction as a potentially informative approach to understanding the role of epigenetic regulation in oral carcinogenesis.

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BACKGROUND & INTRODUCTION

"The emperor of all maladies" in the oral cavity

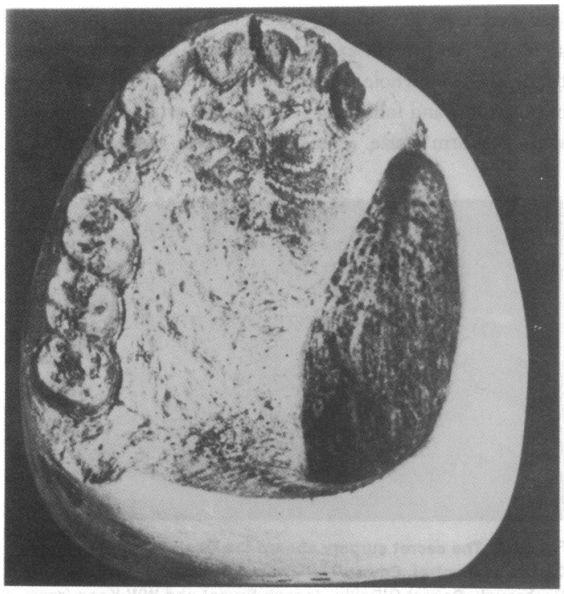
The definition of cancer was recently well documented by Dr. Siddhartha Mukherjee in his 2011 Pulitzer prize winner masterpiece entitled 'The Emperor of all Maladies: a Biography of Cancer." Mukherjee, a noted physician-author, who battles cancer in our sisters institution of the Massachusetts General Hospital and the Dana Farber Cancer Institute, defines this deadly condition as not one disease but many diseases that are in the words of the author: "lethal shape-shifting entities imbued with such penetrating metaphorical, medical, scientific and political potency, that it is often described as the defining plague of our generation"[1]. The oral cavity is no exception; in the United States, cancers of the oral cavity and oropharynx, collectively, are the 8th most common type of malignancy in males and about the 11th in females.

Head and neck cancers are a heterogeneous group of neoplasms that develop from the mucosa of the paranasal sinuses, nasal and oral cavities, larynx and pharynx. Most of these cancers represent epithelial malignancies classified generally as squamous cell carcinoma of the head and neck [2]. Worldwide, cancers of the oral cavity together with lip cancer represent the 8th most common malignancy in males. It is the most common neoplasm seen in males in South East Asia and Papua New Guinea [3].

In 2015, there will be an estimated 45,780 new cases of oral cancer in the United States and 8,650 people will die from this disease [4]. Insight into how common oropharyngeal squamous cell carcinoma may be appreciated is through the fact that of forty-four presidents of the United States of America two have been afflicted with oral squamous cell carcinoma. One was General Ulysses S. Grant who developed squamous cell carcinoma of the right tonsillar fossa, and the other was Grover Cleveland **(Figure 1)**, who had oral squamous cell carcinoma of the left palatal mucosa. President Cleveland's history is worthwhile mentioning because the diagnosis of his squamous cell carcinoma was kept as a secret, to be only revealed in 1971 [5]. Apparently, his oral neoplasm was successfully removed in a secret surgery on board of a yacht. He was able to serve his full term and died on June of 1908. The cause of his death reported in the death certificate was not very clear. It mentioned that he died of heart failure,

complicated by pulmonary embolism and edema. Others thought he died of intestinal obstruction. Nevertheless, his oral cancer is thought to be of verrucous type of squamous cell carcinoma, because of the indolent behavior [5]

A



B

Figure 1. (A) Artistic representation of President's Cleveland secret surgery on board of the Oneida yacht, for removal of oral squamous cell carcinoma of the left palatal mucosa. Artist: Chevalier Fortunio Mantania. (Courtesy of Smith, Kline and French Laboratories). (B) Original dental cast of Cleveland's upper jaw taken in 1893 for confection of a maxillary obturator (With permission, and courtesy of the Mutter Museum, College of Physicians of Philadelphia) [5].

Regarding risk factors, tobacco use and alcohol consumption historically have been recognized as major causes of squamous cell carcinoma of the oral cavity [6, 7]. However, for cancers in the oropharynx, currently it is well known that chronic alcohol and tobacco consumption are not the main causative agents. Instead, high risk subtypes of human papilloma virus, specifically, HPV-16, have become recognized as the main inducers [8, 9]. Therefore, it is apparent that there are different molecular and biologic bases for oral versus oropharyngeal squamous cell carcinoma [6].

Incidence rates for oral cavity cancer have decreased over the past 30 years in the United States [10]. Nevertheless, there has been little to no improvement in the overall 5-year survival rate. Some studies, however, are showing overall mortality to have improved slightly, to about 60% (versus 50% some decades ago). Moreover, the survival

rate differs according to staging at tumor diagnosis. There is an approximate 83% 5-year survival rate for stage I cancers, whereas the survival rate decreases to 18% for stage IV neoplasms [4]. Therefore, early detection of tumors is imperative to decrease both mortality and morbidity.

Although it may seem that oral lesions can be readily detected in the mouth which is easily accessible for clinical examination without major effort or inconvenience to the patient or the clinician, screening for potentially malignant oral lesions is typically confounded by difficulty in discriminating between reactive/inflammatory processes versus those that are premalignant and that will likely progress to oral squamous cell carcinoma (oSCC). These oral premalignant lesions mainly include those that clinically present as leukoplakias and erythroplakias, as well as oral sub-mucous fibrosis, palatal lesions in reverse smokers, and the still questionable oral lichen planus [11]. Of these, leukoplakia is the most common precursor lesion of oSCC [12].

In 2005, the World Health Organization (WHO) defined leukoplakia as "a white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer" [3, 11]. Leukoplakias can be further clinically sub-classified into localized and proliferative (based on the degree of mucosal involvement) and into homogenous or non-homogenous (based on the morphology of the lesions). The localized subtype involves one site, is more commonly seen in men, and is strongly associated with smoking. Proliferative leukoplakias are multifocal lesions, which tend to affect multiple contiguous or non-contiguous sites. They are more commonly seen in non-smoking women and almost always progress to oral squamous cell carcinoma over a variable period of time (usually 10 to 20 years) [12]. Homogenous leukoplakias present as white, thin, sometimes fissured plaques that are almost always well demarcated from the adjacent mucosa. Conversely, non-homogenous leukoplakias present as verrucous or nodular lesions that may show areas of erythroplakia and have a higher frequency of developing epithelial dysplasia and oral squamous cell carcinoma [12].

As mentioned earlier, leukoplakias can clinically mimic lesions that are reactive in nature (such as frictional keratosis, and benign morsicatio mucosae oris) or may represent oral epithelial dysplastic lesions, which are premalignant in nature. Hence, a biopsy is necessary for histopathologic evaluation of all suspicious lesions.

The histopathological diagnosis of an oral epithelial dysplastic lesion is based on cytomorphologic and architectural changes, as well as on the disarray in the maturation pattern of keratinocytes. However, standardized criteria for diagnosis are controversial and subjectivity accounts for interpretative variations that have been documented [13, 14].

In addition, there are no predictive factors or established criteria to identify those premalignant lesions that will progress to oSCC, versus those that will remain stable or perhaps even regress. Indeed, several studies have indicated different behaviors of oral dysplastic lesions. *Mehanna et al.* performed a systematic review with meta-analysis and found that oral dysplasias had a 12% rate of malignant transformation and that the mean time for malignant transformation was 4.3 years [15]. Moreover, *Holmstrup et al*. reported that the only significant factors associated with malignant transformation of premalignant lesions were the type of lesion, (i.e., homogenous leukoplakia, non-homogenous leukoplakia or erythroplakia with the latter having the higher risk (90%) for malignant transformation), and the size of the lesion. The authors further observed that other findings, such as surgical intervention, smoking, grades of epithelial dysplasia, demarcation and site had no significance in relation to subsequent development of cancerous lesions [16].

Accordingly, the development of predictive biomarkers and/or molecular-based approaches that could be used for the early recognition and classification of these potentially malignant lesions would be of great help in the detection and prognostication of oral cancer, as well as aid in the identification of more targeted types of interventional procedures [6]. Likewise, animal models for oSCC are critical to our development of biomarkers as well as to furthering our understanding of the genomic and epigenomic basis for oral carcinogenesis.

4-Nitroquinoline-1-oxide induced murine oral carcinogenesis

To this end, it is well known that animal models, specifically murine models, are widely used to carry out studies that will aid in the development of biomarkers as well as therapeutic agents. The first carcinogens that were used in an effort to induce oral carcinogenesis in mice included cigarette smoke and coal tar [17]. However, these failed

to produce tumorigenesis, or in some cases tumor incidence was very low [18]. Later, a polycyclic aromatic hydrocarbon that consisted of 9,10-dimethyl-1-2-benzanthracene, better known as DMBA, was used to induce oSCC in hamster cheek pouch. This chemical is commonly used to stimulate oncogenesis in many other tissues, and indeed works very well inducing tumors in the mouth.

4-Nitroquinoline-1-oxide (4NQO) is a synthetic carcinogen that produces histological and clinical changes comparable to the development of oral squamous cell carcinomas in humans, including the development of dysplastic precursor lesions. In addition, it's molecular effects in cells are very similar to those produced by the ingredients found in tobacco (nitrosamines and benzopirenes), which is the major risk factor for oral cancer. Hence, 4-NQO induced oSCC in mice appears to be an adequate model for evaluating premalignant dysplastic lesions and SCC of the oral mucosa.

Mechanistically, 4NQO is soluble in water and causes extensive cellular oncogenic damage mediated by intracellular oxidative stress, through the production of reactive oxygen species (hydrogen peroxide and superoxide radicals), as well as by the production of metabolites that will bind to DNA preferentially at guanine [18, 19]. More specifically, the oncogenic effects of 4NQO begin with the enzymatic reduction of its nitrogen group, where 4NQO is reduced to 4-hydroxyaminoquinoline-1-oxide (4HAQO) by 4NQO nitro-reductase (NADH) and a quinone reductase (NADPH). 4HAQO is a carcinogenic metabolite implicated in the formation of DNA adducts [20]. In vivo experiments and nuclear magnetic resonance studies have shown that 4HAQO binds preferentially to guanine residues, resulting in guanine to pyrimidine substitution, hence producing mutations in the DNA [21, 22] **(Figure 2)**.

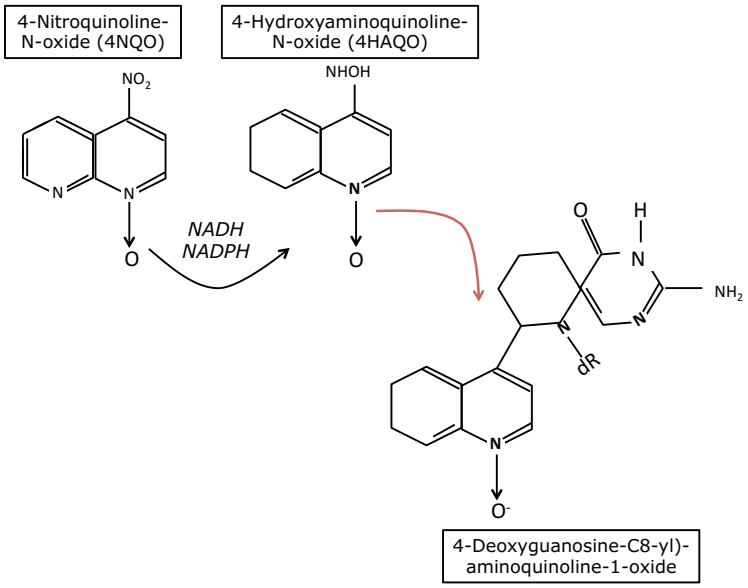


Figure 2. Structure of 4NQO and its carcinogenic metabolite 4HAQO. Subsequent binding of 4HAQO to guanine residue of DNA forming DNA-guanine adducts. Adapted from Kanojia, D. and M.M. Vaidya, *4-nitroquinoline-1-oxide induced experimental oral carcinogenesis.* Oral Oncol, 2006. **42**(7): p. 655-67.

Cancer: a genetic and epigenetic disease

Currently, there is compelling evidence to support the fact that cancer has a genetic and epigenetic origin. Genotype alterations may occur as a result of environmental agents, such as viruses or chemical products; be inherited in the genome itself or result from spontaneous mutations leading to impairment of biologic function. Initial alterations that occur in the DNA and associated molecules are inherited by other cells (daughter cells), leading to an initial tumor or mass where all cells share the same mutations that were present at the initial moment of transformation. Many other additional mutations may develop subsequently, and these may differentially affect individual tumor cells to produce heterogeneous mutational profiles. Hence, carcinogenesis develops as a consequence of accumulation of mutations and tumor virulence may be accelerated by acquisition of additional mutations over time [23].

With regard to the genetic alterations, the principal genes that are affected in neoplastic processes are the growth promoting “proto-oncogenes”; those that inhibit growth of tumors, called “tumor suppressor genes”; those that regulate programmed cell death; and those that are involved in DNA repair [23]. Specifically, in HPV-negative tumors of the head and neck, it has been found that tobacco causes universal loss of function of Tp53, somatic mutations and CDKN2A inactivation. In addition, there are frequent copy number alterations, such as amplification of 3q26/28 and 11q13/22 [24]. Tp53 is affected in 60-80% of squamous cell carcinomas of the head and neck [25]. It encodes the protein p53, which is implicated in the repair of replication errors and damage of DNA [26].

CDKN2A is a gene that encodes the protein $p16^{INK4A}$ and it is located on chromosome 9p21. This protein regulates the cell cycle by inhibiting the function of cyclin-D CDK4/6 that phosphorylates the retinoblastoma protein (pRb). When pRb is phosphorylated, it is released from the pRb/E2F complex and therefore causes the activation of E2F transcription factors, with subsequent progression of the cell cycle into the S phase. Therefore, the expression of $p16^{INK4A}$ mediates senescence and differentiation [26, 27].

Importantly, it has been found that a subgroup of squamous cell carcinomas of the oral cavity that usually show favorable prognosis exhibit a characteristic three-gene pattern of mutations including activating mutations of the HRAS and PIK3CA genes, and inactivating mutations of CASP8, NOTCH1 and Tp53. In addition, this group infrequently exhibits copy number alterations which, as mentioned earlier, are often seen in squamous cell carcinomas affecting the head and neck region[24] . It is pertinent to note that Tp53 mutations are not characteristic of HPV-associated SCC. This subgroup of virally-induced oropharyngeal squamous cell carcinomas is associated with mutations of the oncogene PIK3CA, loss of TRAF3, and amplifications of the gene E2F1 which is also involved in the regulation of the cell cycle [24].

Recent data indicate that in addition to those genetic changes that contribute to the initiation and propagation of tumors, epigenetic alterations also are associated with the development of neoplasms and many other diseases.

Conrad Waddington is considered by many to be the “father of epigenetics”. He was a developmental biologist from the United States of America who was the first to coin the term “epigenetics” in 1950. He defined this new field as molecular alterations that cause potentially heritable changes in cellular phenotype and gene expression, without causing changes in the DNA sequence [28]. Currently, epigenetics is commonly used to define the study of chromatin [29]

The epigenome works via various mechanisms, including regulated modifications to DNA and histones by chromatin-modifying enzymes that transform the chromatin structure through alterations of non-covalent interactions within and around nucleosomes. Another mechanism of epigenetics is the regulation of mRNA translation via non-coding RNAs (ncRNA), which are RNA molecules transcribed from DNA that do not encode proteins [30]. However, ncRNAs are important for other biologic functions, including the control of chromosome dynamics, splicing, ncRNA editing, translational inhibition and mRNA destruction. In the field of epigenetics, ncRNA work in chromatin remodeling and epigenetic memory [31]. All these pathways can regulate DNA associated processes, such as transcription, repair and replication, ultimately leading to the activation or deactivation of genes [30, 32].

In contrast to the genome, modifications in the epigenome are *reversible*, and thus for potentially therapeutic purposes represent a more feasible alternative to the prospect of repairing mutations affecting the genetic code itself. Currently there is information available on three DNA modification pathways associated with epigenetics, namely: DNA methylation, histone modification and ncRNA regulation. DNA methylation and hydroxymethylation, as well as histone acetylation, are the most common epigenetic pathways affected in cancer [30, 32]. DNA methylation mainly occurs in promoter regions, centromeres, telomeres, inactive X chromosomes and repeat sequences [33]. Methylation of the carbon 5-position of cytosine to form 5- methylcytosine (5-mC) that occurs in CpG islands was the first epigenetic modification described in DNA, as well as the most extensively studied pathway in cancer cells. CpG islands are regions enriched with CpG repeats. Each CpG consists of a dinucleotide sequence where cytosine precedes guanine on the ipsilateral strand, and are linked by a phosphodiester bond. Most studies support the fact that CpG islands are present in about 40 to 70% of all mammalian promoters and have an important role in malignant transformation, again, making them

attractive targets for therapeutic purposes [33, 34]. Still, the functional relevance of the methylation patterns of CpG dinucleotides and CpG islands has not been established. Some authors associate promoter methylation with gene silencing [35], while others suggest that methylation outside promoter regions and within gene sequences is associated with gene transcription [36].

Likewise, the exact mechanism by which the process of methylation occurs remains obscure. Nonetheless, there is information on the enzymes which regulate the methylation of cytosine, which are known as DNA methyltranferases (DNMTs) [37]. Three of these have already been identified: DNMT1, DNMT2 and DMNT3. Their roles include, but are not limited to, cellular differentiation, propagation of methylation patterns, genomic imprinting, transcription repression of retrotransposons (a group of genes that travel around different locations causing mutations in genes influencing RNA function); and X-chromosome inactivation [30, 38].

DNA methylation is not the only chromatin modification. There is an active process in which methylation and demethylation both occur to affect biologic functions. This dynamic nature of DNA methylation has been supported by recent studies that showed genome-wide mapping of these alterations in pluripotent and differentiated cells, implying that there is an enzymatic machinery in mammalian cells capable of inducing or preventing the methylation patterns [33].

DNA methylation in oral cancer

Changes in methylation patterns of DNA, including genome wide hypomethylation and promoter region hypermethylation are closely related to the development of oral squamous cell carcinomas. Global hypomethylation has an oncogenic potential through different mechanisms. It causes enhancement of chromosomal instability by the reduction of methyl groups at DNA repetitive elements, which are distributed along the genome. In addition, hypomethylation can re-activate methylation-silenced promoter regions of various proto-oncogenes that at one point were activated and became silenced with progression of human evolution. Moreover, DNA hypomethylation can also reactivate naturally methylated genomic imprinting ("mono-

allelic gene expression due to the inactivation of either the maternal or the paternal allele of a particular locus" [39]) and hence alter gene expression [40].

Likewise, the tumorigenesis potential of promoter hypermethylation in the oral mucosa is mediated by aberrant methylation in CpG-rich islands present in promoter regions of various tumor suppressor genes. This results in closed chromatin, inhibiting the interaction of transcription factors that bind to promoter regions of tumor suppressor genes, resulting in transcriptional silencing.

Li et al. reported that DNA methylation patterns were significantly higher in genes associated with oral SCC, when compared to normal mucosa. Using pyrosequencing methylation assays they demonstrated that methylation levels of selective genes including FLT4, KDR and TFPI2 were higher in oSCC samples and that this could serve as a potential biomarker [41]. Along the same lines, *Melchers et al.* also showed that hypermethylation of DNA was implicated in the metastatic potential of tumor cells in oSCC. They evaluated the methylation pattern of specific genes that are involved in the development of nodal metastases using Methylation Specific PCR Primers. Their results suggest that multiple genes were hypermethylated, including OCLN, CDKN2A, MLH1, MGMT, and DAPK1 (although only the last two were also associated with positive nodal status [42]).

Loss of 5-hydroxymethylcytosine and cancer development

It has been shown that the enzymatic oxidation of 5-mC to 5-hydroxymethylcytosine (5-hmC) may act as a stable modification of DNA and that subsequent removal of 5-hmC involves a complicated process of epigenetic regulation [43]. 5-hmC can both activate transcription of genes, as well cause gene silencing. It is the most copious intermediate of the active DNA demethylation pathway. In addition, its presence directly correlates with the differentiation stages of various human tissues [44-47]. *Haffner et al.* recently demonstrated that 5-hmC was abundant in the majority of human and mouse embryonic and adult tissues. Interestingly, in mouse embryos and adults, there was a strong association between the expression of 5-hmC and the differentiation state of the cells in colon, cervix, skin and dorsal tongue. In these tissues, there was a hierarchical distribution of 5-hmC expression. In squamous epithelium, the basal cell layer showed very low levels of 5-hmC, with higher levels in the uppermost

layers of the epithelium. Similar results were demonstrated in human colonic mucosa, where the apical more differentiated cells showed strong 5-hmC expression, and more undifferentiated cells at the bases of the crypts showed reduced 5-hmC levels [47].

Furthermore, there are three enzymes, known as the mammalian 5-mC dioxygenases, that catalyze the conversion of 5-mC to 5-hmC. [48]. These enzymes are part of a group of ten eleven-translocation (TET) enzymes, which received their name based on a recurrent chromosomal translocation t(10;11)(q22;q23) [49], that apposes the *MLL* gene with *TET1* in a group of patients affected with acute myeloid leukemia [50]. 5-mC dioxygenases can oxidize both DNA and RNA that was previously methylated on either the carbon or nitrogen of the base.

Similar to 5-hmC, the TET family of enzymes can have both activating and repressive functions [44], but most importantly they are responsible for demethylation of DNA. There is scant information about the exact mechanisms of how DNA demethylation occurs. It has been stipulated that this process is initiated after the sequential oxidation of the methyl group of 5-mC and withdrawal of the final modified group by thymine DNA glycosylase, with subsequent base excision repair pathway to release cytosine from 5-mC [51, 52]. Moreover, the TET family enzymes require the Fe(II)/α ketoglutarate-dependent oxygenase enzymes to perform these demethylation junctions [43, 44] **(Figure 3)**.

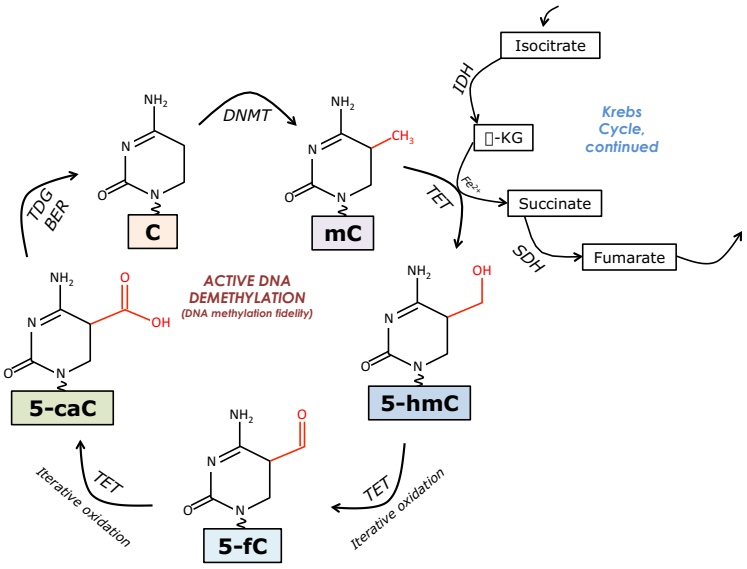


Figure 3. Adapted from *Lee JJ et al* [30]. This process allows the removal of a methyl group from 5-methylcytosine via oxidation of this methyl group by the (TET) family enzymes, which requires a a-ketoglutarate, that is a Krebs cycle intermediate.

TET's ability to induce the removal of methyl groups from the DNA makes it responsible for orchestrating the DNA demethylation rescue, becoming the "guardian of CpG islands" [53]. Therefore, alterations or loss of TET function can correlate with ominous biologic consequences. For instance, TET2-deficient mice develop chronic myelomonocytic leukemia, which correlates with the high prevalence of this mutation in many patients affected with this disease. In addition, previous studies suggest that the mutations associated with TET2-mediated oncogenesis are mostly loss-of-function mutations that lead to elevated 5-mC levels and decreased or sometimes loss of 5-hmC expression within neoplastic cells. Likewise, mutations in TET2 seem to enhance the self-renewal potential of malignant cells [54].

Importantly, loss of 5-hmC has been reported in many malignant neoplasms, including melanoma, breast cancer, gastrointestinal stromal tumor, hepatocellular carcinoma and oral squamous cell carcinoma [55-58].

Recently, *Lian et al.* [58] in the Program in Dermatopathology of the Brigham and Women's Hospital Department of Pathology, demonstrated that 5-hmC is lost in melanomas and that this is associated with increased tumor virulence and poor prognosis. In contrast, expression of 5-hmC was strongly retained in benign nevi, suggesting that its loss could represent an epigenetic hallmark of melanomas with paramount diagnostic and prognostic significance. Moreover, the investigators revealed that by overexpressing TET2 in a mouse-human melanoma xenograft model or by overexpressing the gene encoding isocitrate dehydrogenase 2 (IDH2)[i] in a zebrafish model, melanomas were less invasive and had a more indolent behavior.

In addition, *Larson et al.* ([59] from the same group), showed a progressive gradient of loss of 5-hmC from benign dermal nevi to high-grade dysplastic nevi and melanomas. The authors demonstrated that benign nevi homogenously express high levels of 5-hmC, whereas dysplastic nevi exhibit partial loss and melanomas exhibit complete loss of the biomolecule. Likewise, this partial or complete loss of 5-hmC represents a deficiency in TET family of active DNA de-methylation enzymes.

To date, only one study has investigated the association between 5-hmC and

[i] *IDH2* overexpression is thought to restore the "5-hmC landscape" by enhancing TET's functions.

oSCC (*Jawert et al [55])*. With immunohistochemical studies, the authors demonstrated a significant decrease in 5-hmC and TET2 expression in oSCC, when compared to healthy oral mucosa [55]. However, no information was provided in regards to 5-hmC in oral premalignant dysplastic lesions or in benign hyperkeratotic and inflammatory oral mucosa lesions, and mechanistic insights or experimental approaches were lacking.

The studies above suggest that 5-hmC immunoreactivity could potentially serve as a diagnostic tool to distinguish between benign and malignant oral epithelial lesions, and perhaps aid in the distinction between those oral dysplasias that will likely progress to oral squamous cell carcinoma. In addition models for exploration of the functional significance of 5-hmC in the genesis and evolution of oSCC are lacking.

Accordingly, further study of the expression of 5-hmC in oral premalignant lesions and oral squamous cell carcinoma represents an opportunity to better understand a novel epigenetic molecular pathway that may serve as a biomarker for early diagnosis of oral neoplasms and subsequent improvements in the prevention and therapy for oral cancer. Our approach will deploy specific methods intent upon generating new information about the pathogenesis of oSCC with respect to epigenetic regulation, thus also providing important data germane to development of novel therapeutic strategies aimed at normalizing the epigenetic landscape in oSCC.

OBJECTIVES

The overall objectives of this research project consist of: **[1]** To evaluate the patterns of 5-hmC expression in benign, reactive, inflammatory/immune mediated and neoplastic lesions affecting the human oral mucosa, including: non-inflamed/non-cancerous mucosa at the edges of fibromas and reactive frictional keratotic lesions; oral lichen planus; moderate to severe oral epithelial dysplasia, and oral squamous cell carcinoma. **[2]** To confirm expression and genomic profiles of 5-hmC using primary oral keratinocyte cell lines (OKF4), immortalized normal oral keratinocyte cell lines (OKF4/TERT-1 and OKF4/E6E7), moderate to severe oral dysplasia cell lines (POE9n), immortalized moderate to severe oral dysplasia cell lines (POE9n/TERT), and immortalized oral squamous cell carcinoma cell lines (SCC15, SCC25, SCC68, SCC71).

[3] To compare the findings of 5-hmC expression in human oral mucosa to an established murine model of oral carcinogenesis elicited by 4-nitroquinoline-1-oxide (4NQO).

MATERIALS AND METHODS

Studies of discarded bioarchives of de-identified patient specimens (tissue blocks) were approved by the Institutional Review Board of the Brigham and Women's Hospital (Boston, MA). These bioarchives of de-identified patient specimens were supplied courtesy through Dr. Sook-Bin Woo, who is an Associate Professor at the Harvard School of Dental Medicine and Co-Director of the Center for Oral Pathology, StrataDx (Boston, MA).

Clinical data acquisition, immunohistochemical studies and semi-quantitative analysis of human tissue

A total of 66 human cases (N=66) were evaluated: Nine cases diagnosed as fibromas (F, n_F=9). These fibromas will serve to evaluate the pattern of 5-hmC expression in the non-inflamed/ non-cancerous mucosa at the margins of excisions; nine cases diagnosed as frictional keratosis (FK, n_{FK}=9); ten cases diagnosed as lichen planus (LP, n_{LP}=10); Fifteen cases diagnosed as moderate-to-severe oral epithelial dysplasia (OED, n_{OED}=15) and twenty-three cases diagnosed as oral squamous cell carcinoma (oSCC, n_{oSCC}=23). Cases were identified in a 7-month period (January 2015 through July 2015) from the archives of Strata Pathology Services, the surgical pathology laboratory affiliated with the Harvard School of Dental Medicine in Boston, MA, USA.

Clinical data was obtained when available. Lesions were present in the alveolar mucosa; gingiva; buccal mucosa; hard and soft palatal mucosa; dorsum, ventrum, lateral and base of tongue; and floor of mouth **(Table 1)**. Hematoxylin and eosin (H&E) sections and prior diagnoses were independently reviewed and confirmed by two oral pathologists (MCCN and SBW).

Immunohistochemistry for 5-hydroxymethylcytosine (5-hmC) was performed on all cases of fibromas, oral frictional keratosis, oral lichen planus, moderate to severe oral

epithelial dysplasia, and oSCC as previously described by Lian *et al* [58]. Briefly, sections were incubated overnight with rabbit-anti-5-hmC (Active Motif, Carlsbad, CA; 1:5000 dilution), washed, and subsequently incubated with a peroxidase-linked anti-rabbit IgG (Vector Laboratories, Burlingame, CA; 1:200 dilution). The sections were then treated using the corresponding hydrogen peroxide substrate kit (Vector Laboratories, Burlingame, CA) and counterstained in hematoxylin and clarifying solution (Fisher Scientific Company, Kalamazoo, MI). Appropriate isotype-matched antibody controls and tissue controls were included for all experiments. TET-2 overexpressed melanoma cells were used as control tissue.

5-hmC staining was scored in accordance with previously published methodology [59]. In summary, immunoreactivity was estimated based on the nuclear staining intensity (0-4; 0 = absent; 4 = dark brown reactivity involving the entire nuclear profile, and 1 through 3 representing semiquantitative intermediates [1 = faint tan; 2 = light brown; 3 = medium brown]. Likewise, the percentage of 5-hmC-positive cells keratinocytes, as assessed over representative 1- mm^2 fields, was also determined. Fields were selected based on the presence of key histologic features relevant to the study (i.e. areas of dysplastic and poorly differentiated cells), as assessed and determined by H&E examination alone. A total of two randomly selected and representative sections were examined in terms of nuclear staining positivity and intensity in all histologic sections.
An immunoreactivity score (IRS) was derived by multiplying the percentage of positive cells with the number consistent with staining intensity. The resultant scores were analyzed statistically among specific foci of interest (i.e., normal and inflamed oral mucosa, dysplastic oral mucosa and cancerous oral mucosa, etc.) by performing one-way ANOVA analysis and Tukey test, using GraphPad Prism version 6 (GraphPad Software, La Jolla, CA). All p-values were two-tailed, with a p-value < 0.05 considered statistically significant.

All semiquantitative immunoreactivity scoring was performed by one investigator (MCN). In the first session, the investigator (MCN) reviewed all cases. In addition, this same investigator (MCN) reviewed a selective subset of cases for a second time (session 2), one month apart, to ensure intra-observer reliability.

A random, selective subset of cases, including fibromas, frictional keratosis, oral lichen planus, moderate to severe oral dysplasia and oral squamous cell carcinoma were reviewed by another investigator (GFM), to ensure concordance and inter-observer reliability.

The resultant scores of the two readings of the first investigator and of the two investigators were analyzed statistically among three parameters (i.e. percentage of positive cells, intensity of the staining and product of these two attributes) by performing T-test, using GraphPad Prism version 6 (GraphPad Software, La Jolla, CA). All p-values were two-tailed, with a p-value < 0.05 considered statistically significant.

Table 1. Summary of clinical data.

	Normal Mucosa	Frictional Keratosis	Lichen Planus	Epithelial Dysplasia	Oral Squamous Cell Carcinoma
Gender	M: 45% (4)	M: 55% (5)	M: 60% (6)	M: 60% (9)	M: 57% (13)
	F: 55% (5)	F: 45% (4)	F: 40% (4)	F: 40% (6)	F: 43% (10)
Location	Tongue: 100% (9)	Tongue: 45% (4) Ridge: 55% (5)	Buccal mucosa: 80% (8) Alveolar mucosa: 20% (2)	Tongue: 80% (12) Floor of mouth: 20% (3)	Tongue: 72% (17) Gingiva: 9% (3) Floor of mouth: 9% (3)

Acquisition of cell lines and cell cultures

Cell lines were provided courtesy of Dr. Matthew Ramsey, from the Cell Culture Core Collection (Brigham and Women's Hospital Department of Dermatology). Table 2 shows characterization of these cell lines **(Table 2)**. Cells were cultured in GIBCO keratinocyte serum-free medium (K-sfm) (Invitrogen/Life Technologies) with 12.5 μg/mL bovine pituitary extract, 0.2 ng/mL epidermal growth factor (EGF), and CaCl2 to a final Ca2+ concentration of 0.4 mmol/L, as described previously [60, 61]. After cultures reach ~60% confluence, they were daily fed with the (K-sfm) medium at a concentration of 1:1Ksfm:DF.k.

DNA isolation and dot blot

Genomic DNA was isolated from cultured cells using standard methods (Gentra Puregene QIAGEN Gaithersburg, MD). Samples were lysed in a buffer solution composed of proteinase K, 100nM Tris-HCL, pH8.5, 5 mM EDTA, 0.2% SDS, 200 nM NaCl (55°C overnight), and were subsequently extracted with phenol:chloroform:isoamyl alcohol (25:24:1 Saturated with 10nM Tris, pH 8.0, 1nM EDTA) and precipitated with 2 volumes (100% ethanol and 1/10 volume 3 M sodium acetate). The genomic DNA was then recovered and dissolved with TE buffer (10 nM Tris-HCL, pH8.0, 1nM EDTA). The DNA concentration was determined with NanoDrop 1000 (Thermo Scientific). Genomic DNA samples were further shared with needle at ~500ng/µl concentration.

The dot blot was performed using a Bio-Dot Apparatus. In summary, the DNA was loaded in a 96-well plate in 30 µl TE buffer with 2-fold serial dilution (100ng- 1µg), mixed with 20 µl 1 M NaOH and 25 nM EDTA. The plate was then sealed and heated at 95°C for 10 minutes. After heating, the plate was immediately cooled down on ice, and 50µl ice-cold-2M ammonium acetate (pH 7.0) was added. The plate was placed on ice for 10 minutes.

The nitrocellulose membrane was incubated with ddH2O for 20 minutes and then in 6X SCC for 20 minutes. Subsequently, the membrane was rehydrated on the Bio-Dot Apparatus with TE buffer, and the denatured DNA sample was applied and then washed by 2X SCC. The membrane was rinsed by 2X SCC, and allowed to air-dry. The completely dried membrane was baked for 2 hours at 80°C and then blocked with TBS-T containing 5% non-fat milk for 1 hour at room temperature.

The membrane was incubated with primary 5-hmC antibody (1:1000) for 1 hour at room temperature. The membrane was then washed with TBS-T three times, and incubated with HRP (enzyme horseradish peroxidase) conjugated secondary antibody (1:10000) at room temperature for 1 hour. The signal was developed with ECL after washed with TBS-T.

Then, the dot blot was analyzed using Image Lab^{TM} Software. This software measured the integrated density of 5-hmC in each dot. The machine created a circular selection that was then dragged over the first dot, and then repeated for each of the other dots. Later, the calculation was done and the mean of the DNA density for each dot was

obtained for each of the concentrations of DNA (0.5µg, 0.25µg and 0.125µg). Methylene blue was used for DNA concentration loading control. Statistical analysis of the results was performed by one-way ANOVA analysis using GraphPad Prism version 6 (GraphPad Software, La Jolla, CA). All p-values were two-tailed, with a p-value < 0.05 considered statistically significant.

Table 2. Human cell lines

Cell Line	Donor sex (Age in years)	Special characteristics	References
Oral keratinocyte (OKF4/1.3mM Ca) (Floor of mouth)	M,28	Normal non-keratinized oral mucosa from floor of mouth	[48,50]
Oral keratinocyte (OKF4/TERT-1) (Floor of mouth)	M,28	Normal non-keratinized oral mucosa from floor of mouth Immortalized	[60]
Oral keratinocyte (OKF4/E6E7) (Floor of mouth)	M,28	Normal non-keratinized oral mucosa from floor of mouth Immortalized; p53 and pRb- deficient	
Oral keratinocyte from moderate to severe dysplasia (POE9n) (Floor of mouth)	M, 65	Homozygous deletion of $p16^{INK4A}$ and $p14^{ARF}$; p53-deficient	[60, 61]
Oral keratinocyte from moderate to severe Dysplasia (POE9n/TERT) (Floor of mouth)	M, 65	Homozygous deletion of $p16^{INK4A}$ and $p14^{ARF}$; p53-deficient. TERT overexpressed; Immortalized	[60, 61]
Oral keratinocyte from oral SCC-15 (Base of tongue)	M, 55	p16- and p53 deficient; Immortalized	[61, 62]
Oral keratinocyte from oral SCC-25 (Side of tongue)	M, 74	p16- and p53 deficient; Immortalized	[61, 62]
Oral keratinocyte from oral SCC-68 (Base of tongue)	M, no data	p16- and p53 deficient; Immortalized	[61]
Oral keratinocyte from oral SCC-71 (Soft palate)	M, 80	p16- and p53 deficient; Immortalized	[61, 63]

Clinical data acquisition, immunohistochemical studies and semi-quantitative analysis of animal tissue

Animal tissue was received from a collaborative study between the Department of Oral Medicine, Beijing Hospital for Stomatology, School of Stomatology, Capital Medical University, Beijing, China; and the Cancer Research Program, North Carolina Central University, Durham, North Carolina.

For this study an established murine model of oral carcinogenesis elicited by 4-nitroquinoline-1-oxide (4NQO) was used. Ten animal oral tongue samples (N=10) were received as unstained paraffin embedded tissues on glass slides. Five consisted of non-treated normal dorsal tongue mucosa (T, n_T=5) and five consisted of oral squamous cell carcinoma (mSCCn, n_{mSCC}=5). Oral epithelial dysplasia was evaluated at the margins of the oral squamous cell carcinomas. Hence, the five samples used for carcinomas were also examined for oral epithelial dysplasia (OED, n_{OED}=5).

The model consisted of male wild-type C57BL/6J mice (C57BL/6J mice), also known as “C57 black 6” or “black 6” was used. This is an inbred strain produced by a minimum of 20 generations of brother-sister mating. Hence, colonies are genetically identical within each strain). As described by *Guo et al.* [64], oSCC was elicited in these animals by including in their drinking water 4NQO (Sigma) at a concentration of 100 μg/mL, “ad libitum” for a period of 8 weeks, followed by drinking water alone for a period of 16 weeks for a total of 24 weeks. Control mice were given only drinking water “ ad libitum” for 24 weeks.

All animals were sacrificed at 24 weeks after receiving injections of bromodeoxyuridine. The tongue was harvested and the sections that showed macroscopic alterations were fixed in 10% buffered formalin overnight and then processed and embedded in paraffin. Sections were cut at 5 μm and then stained with hematoxylin and eosin.

Histopathologic analysis was performed in a blinded manner, without prior knowledge of the experiment. Mild dysplasia, severe dysplasia, and oSCC were diagnosed as described by *Sun Z et al. [65]*. Briefly, severe dysplasia was diagnosed in the presence of dysplastic alterations including: maturation disarray, increased number of mitotic figures, increased nuclear:cytoplastic ratio, and loss of polarity of basal cells.

oSCC was diagnosed when neoplastic cells were seen infiltrating the underlying connective tissues [64].

Immunohistochemistry for 5-hydroxymethylcytosine (5-hmC) and 5-hmC staining scoring was performed following the same protocols used for human tissues on all animal cases including, non-inflamed/non-neoplastic oral dorsal tongue mucosa, oral squamous cell carcinoma and oral dysplasia present at the edges of the tumors. As mentioned earlier, procedures were based on previously published methodology [58, 59] and as previously described in this manuscript.

All semiquantitative immunoreactivity scoring was performed by one investigator (MCCN) and a random, selective subset of moderate to severe oral dysplasia and oral squamous cell carcinoma cases were reviewed by two other investigators (GFM, CGL) to ensure concordance.

Immunohistochemical staining scores were compared between specific foci of interest (i.e. normal, dysplastic oral mucosa and cancerous oral mucosa, etc.) by performing one-way ANOVA analysis and Tukey test using GraphPad Prism version 6 (GraphPad Software, La Jolla, CA). All p-values were two-tailed, with a p-value < 0.05 considered statistically significant.

RESULTS

I. 5-hydroxymethylcytosine immunoreactivity in human normal and inflamed oral mucosa, oral moderate to severe dysplasia and oral squamous cell carcinoma

The evaluation of the lesions was based on three parameters (percentage of positive stained cells, intensity of the staining and pattern of distribution of the positive stained cells). These were mentioned earlier in this manuscript and are adapted from those previously published in studies regarding 5-hm immunohistochemistry in human and animal tissues [47, 58, 59]. Briefly, immunoreactivity was quantified based on the nuclear staining intensity (0-4; 0 = absent; 4 = dark brown reactivity diffusely obscuring internal nuclear chromatin structure, and 1 through 3 representing semiquantitative intermediates [1 = faint to light brown; 2 = light to medium brown; 3 = medium to dark brown] **(Figure 4A)**. Likewise, the percentage of 5-hmC-positive keratinocytes (of all

intensities) was assessed. These features were evaluated and compiled via examination of representative 1 mm^2 fields. Fields were selected based on the presence of key histologic features relevant to the study (i.e. areas of dysplastic and poorly differentiated cells), as assessed and determined by correlative H&E examination of adjacent tissue sections. An immunoreactivity score (IRS) ***[a x b = c]*** was derived by multiplying the score representative of the percentage of positive cells ***[a]*** with the number derived from staining intensity ***[b]***. The resultant product scores ***[c]*** were analyzed statistically in relationship to the different lesions under study. Importantly, we noted that when comparing the predictive power of ***a*** versus ***b*** alone, ***a*** was expressed relatively constantly (> 75% of all cases exhibited high percentage of positive cells, score 4), with a minority exhibiting scores of 3 or 2. To this end, ***b*** alone led to statistically significant differences that did not deviate from the interpretation derived from use of ***c***, the product of ***a x b*** **(Figure 4B)**.

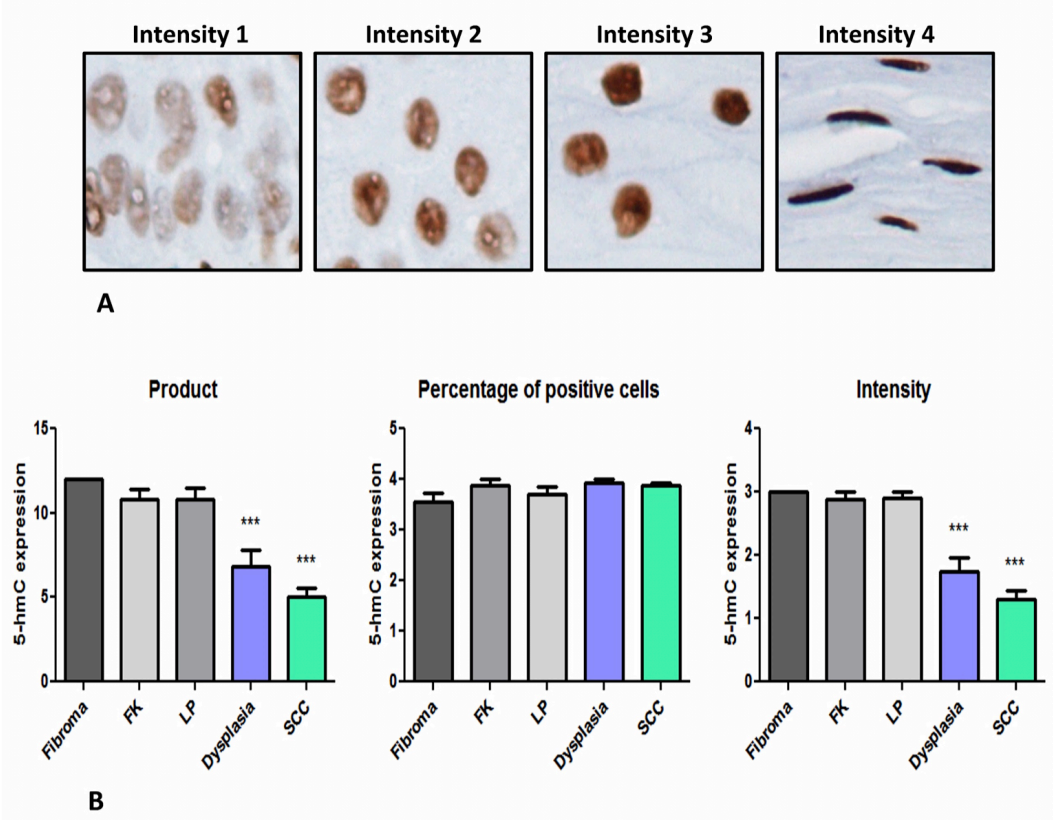


Figure 4. 5-hmC immunoreactivity evaluation system. An immunoreactivity score (IRS) was derived by multiplying the percentage of positive cells with the number consistent with staining intensity. (A) Nuclear staining intensity consists of: 0-4; 0 = absent; 4 = dark brown reactivity diffusely obscuring internal nuclear chromatin structure, and 1 through 3 representing semiquantitative intermediates [3 = medium to dark brown; 2 =

light to medium brown; 1 = faint to light brown]. (B) ANOVA analysis reveals statistically significant differences in 5-hmC immunostaining with respect to nuclear staining intensity and resultant product score between benign reactive lesions (fibroma, FK, LP) with dysplasia and squamous cell carcinoma ($p<0.001$).

Oral mucosa that was non-inflamed and non-neoplastic at the edges of fibromas ($n_F=9$) exhibited strong 5-hmC nuclear immunoreactivity in epithelial cells (mean percentage of positive cells = 4.0; mean IHC intensity = 3.0) **(Figure 5A)**. Similarly, benign reactive lesions of frictional keratosis and immune mediated lichen planus exhibited comparable results. Regions of frictional keratosis cases ($n_{FK}=9$) demonstrated strong 5-hmC nuclear immunopositivity of the epithelial cells (mean percentage of positive cells 3.8; mean IHC intensity 2.8) **(Figure 5B)**. Lichen planus ($n_{LP}=10$) cases showed retention of nuclear 5-hmC (mean percentage of positive cells 3.7; mean IHC intensity 2.9) **(Figure 5C)**.

Interestingly, even though they all exhibited at least 75% stained cells, the percentage of negatively stained cells tended to be higher in LP when compared to fibromas and frictional keratosis. In contrast, moderate to severe oral dysplastic lesions ($n_{OED}=15$) showed loss of intensity of 5-hmC nuclear staining, with mean IHC intensity score of 1.7. However, there was a similar percentage of positive cells (mean percentage of positive cells 3.9) **(Figure 5D)**. Furthermore, oral squamous cell carcinomas ($n_{oSCC}=23$) demonstrated diminished intensity of nuclear 5-hmC immunoreactivity (mean IHC intensity 1.2), but also exhibited high percentage of positive stained cells (mean percentage of positive cells 3.8) **(Figure 5E)**.

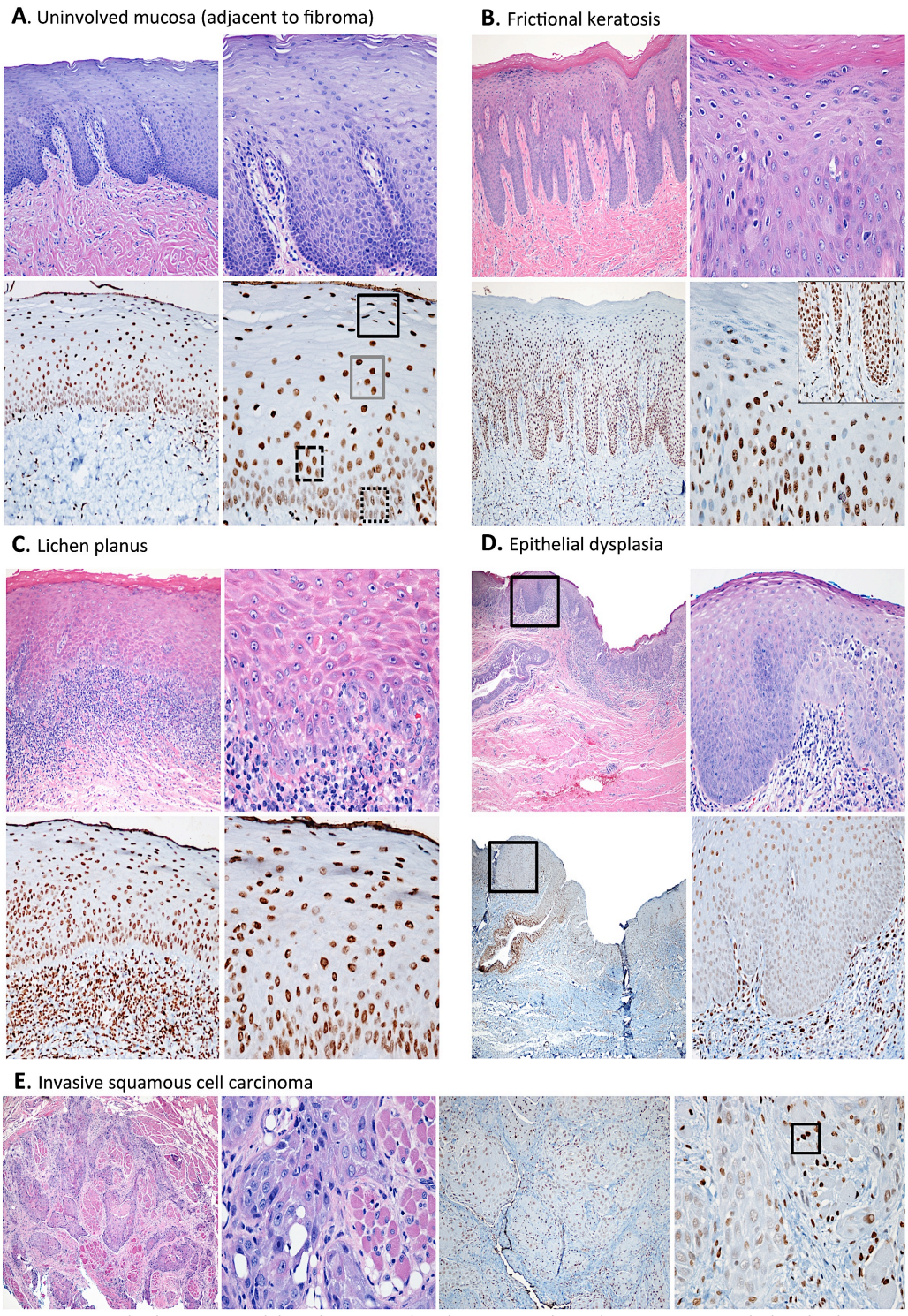


Figure 5. Correlative H&E histology and 5-hmC nuclear immunoreactivity in human oral mucosal lesions. (A) Uninvolved mucosa adjacent to fibroma. Note differences between progressive hierarchical staining intensity (demonstrated by dotted, dashed, solid grey and black outlines). (B) Frictional keratosis (high magnification denoted by inset). (C) Lichen planus (D) Oral epithelial dysplasia (high magnification denoted by black solid outline) and (E) Invasive oSCC (black solid outline denoted skeletal muscle cells stained with 5-hmC that served as internal control) (H&E 200X, 400X and 5-hmC 200X, 400X).

Regarding the percentage of positive cells, there were no statistically significant differences between mucosa at the margins of fibromas, frictional keratosis, lichen planus, dysplasia and squamous cell carcinomas (p 0.1298). In contrast, there were statistically significant differences for the intensity scores for the benign lesions, including fibromas, frictional keratosis and lichen planus, when compared to dysplasia and oral squamous cell carcinoma lesions ($p<0.001$) **(Figure 6A)**. When the product or 5-hmC immunoreactivity score (IRS) was evaluated among all non-dysplastic/cancerous lesions compared to normal mucosa at the edges of fibromas, there were no statistically significant differences between fibromas and the other two benign inflammatory conditions (FK and LP), although there was a statistically significant result when compared to dysplasia and oSCC ($p<0.001$) **(Figure 6B, 6C)**. Finally there were no statistically significant differences in 5-hmC IRS score among oral dysplasias and oral squamous cell carcinomas **(Figure 6D)**.

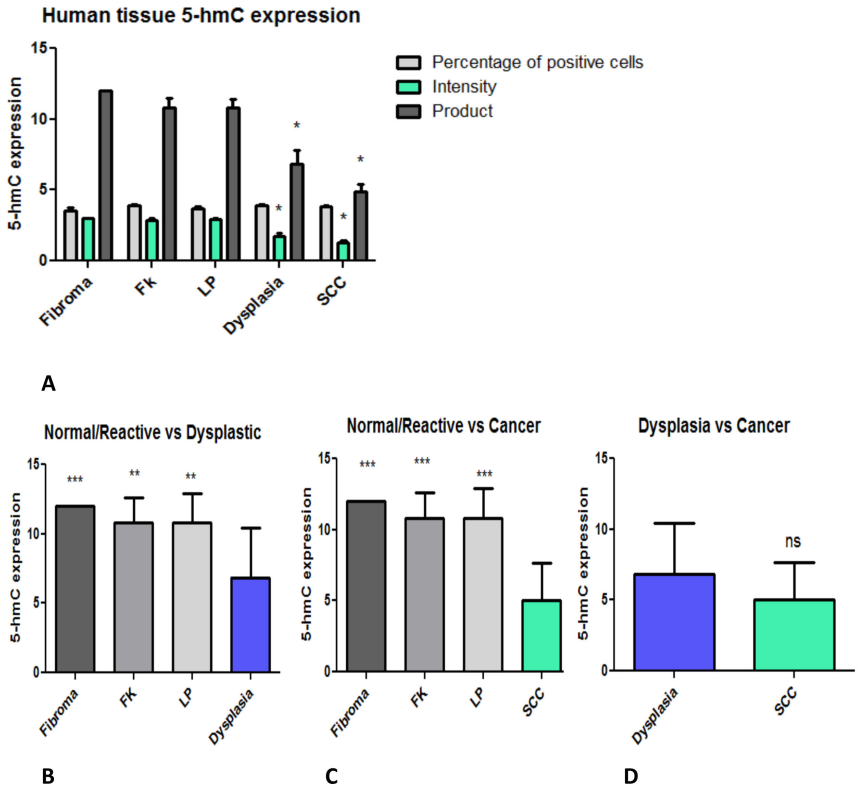


Figure 6. (A) ANOVA analysis reveals statistically significant differences in 5-hmC immunostaining with respect to nuclear staining intensity and resultant (IRS) product score ($p<0.001$). (B & C) When the (IRS) product score was evaluated among all non-dysplastic/cancerous lesions (fibroma, FK, LP) compared to dysplasia and oSCC, there were statistically significant differences ($p<0.001$). Benign lesions retained the strongest nuclear intensity for 5-hmC whereas dysplasia and oSCC demonstrated loss. (D) There were no statistically significant differences in 5-hmC (IRS) product score among oral dysplasias and oSCC.

Distinct architectural patterns of 5-hmC immunoreactivity were observed in all different types of lesions. In non-inflamed and non-neoplastic oral mucosa at the edges of fibromas, 100% of the lesions ($n_F=9$) exhibited a predominant hierarchical pattern of staining, as has been typically seen and described in normal dorsal tongue from murine models, as well as other human epithelial tissues, such as skin and bladder [47, 59]. In these tissues the expression of 5-hmC correlated with the level of differentiation of the cells, where the basal cell layer (immature cells) demonstrated low intensity staining (IHC intensity 1). As cells matured with ascent above the basal layer, the cells gradually and progressively acquired more intense nuclear expression of 5-hmC (**Figure 7A**). Therefore in the stratum spinosum (prickle cell layer) or "middle portion" of the epithelium, the cells exhibited IHC intensity 3. Lastly, the most superficial layer/layers of the epithelium, consistent with the most differentiated cells, exhibited high intensity for nuclear staining (IHC intensity 4).

Similarly, for 10% of FK lesions ($n_{FK}=1$) and 30% of LP lesions ($n_{LP}=3$), a hierarchical pattern of staining was also noted. In contrast, the other 90% of FK lesions ($n_{FK}=8$) and 70% of LP lesions ($n_{LP}=7$) failed to exhibit a hierarchical pattern of nuclear staining. Rather, a haphazard distribution of epithelial cells showing negative and positive nuclear stained cells (IHC intensity 1-3) was detected throughout the thickness of the epithelium. Notably, for LP lesions, the stratum spinosum and corneum (middle and top layers of the epithelium) demonstrated a higher number of negative stained cells **(Figure 7B)**.

Regarding OED, the staining architectural pattern for 73% ($n_{OED}=11$) consisted of diminished reactivity (mean IHC score 1.7) that was uniformly distributed in the lower to mid strata of the epithelium and which correlated with the zones of dysplasia, as evidenced in adjacent, conventionally-stained sections. The other 27% ($n_{OED}=4$)

exhibited a dichotomy in which cells showed light nuclear staining (mean IHC intensity 1), with a minority of cells (about 10%) showing nuclear staining of IHC intensities ranging between 2 to 3. In 85% of oSCC (n_{oSCC}=19) tumor islands exhibit cells with uniform light nuclear staining (mean IHC intensity 1). The other 15% (n_{oSCC}=4) showed a dichotomy in 5-hmC immunoreactivity, where tumor islands consisted of about 50% of cells with light nuclear staining (mean IHC intensity 1) and 50% of cells with nuclear staining of IHC intensities raging between 2 to 3 **(Figure 7B)**.

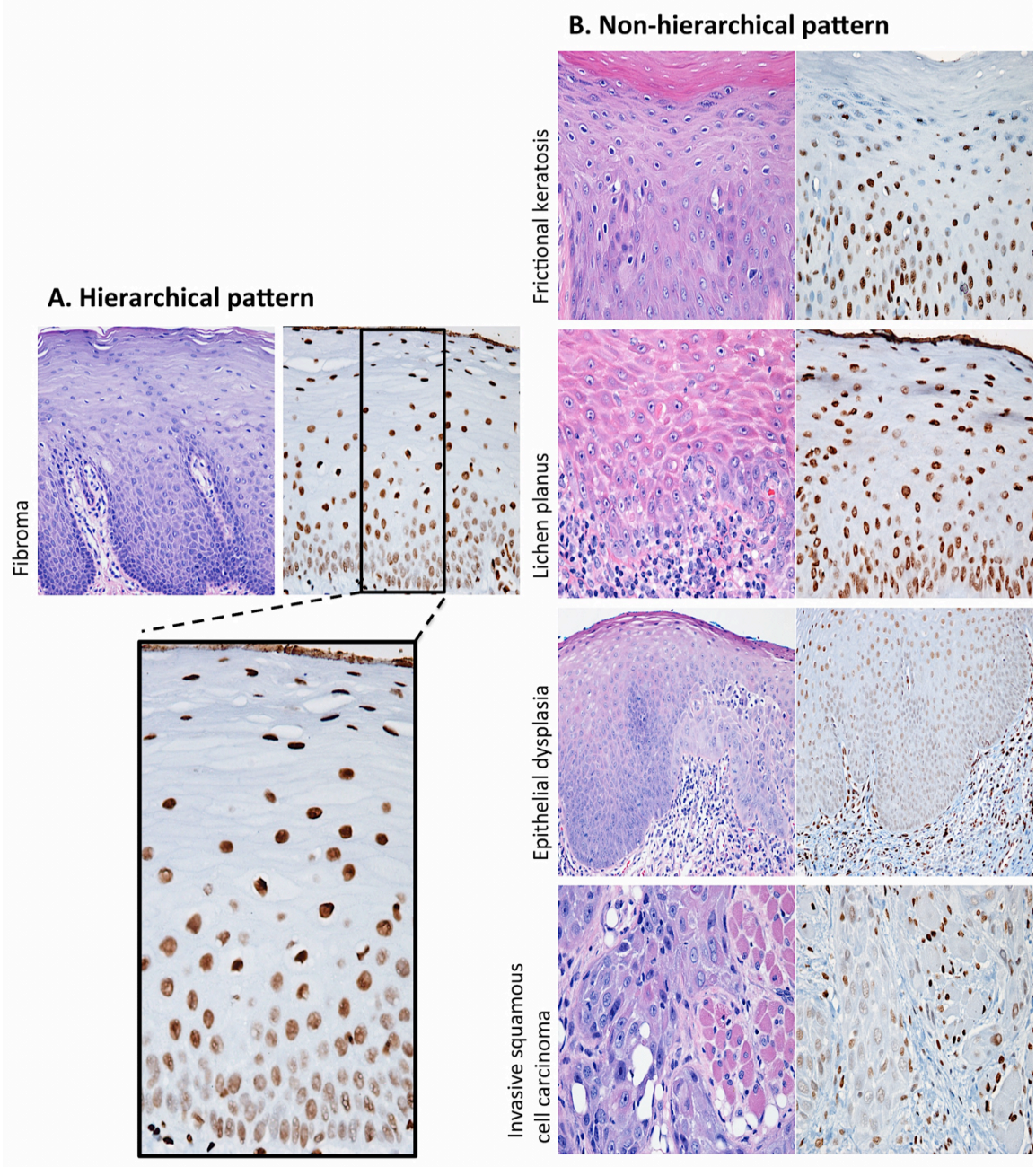


Figure 7. Architectural patterns of 5-hmC immunoreactivity. (A) Fibromas exhibit a predominant hierarchical pattern of 5-hmC immunoreactivity. Insert: The expression of 5-hmC correlates with the level of differentiation of the cells, where the basal and para-basal cells (immature cells) demonstrates low intensity staining (IHC intensity 1). Then, as cells matured with ascent above the basal layer, they gradually and progressively acquired more intense nuclear expression of 5-hmC. In the stratum spinosum (prickle cell layer) or "middle portion" of the epithelium, the cells exhibit IHC intensity 3, and in the most superficial layer/layers of the epithelium (the most differentiated cells) exhibit high intensity for nuclear staining (IHC intensity 4). (B) Frictional keratosis, lichen planus, dysplasia and oral squamous cell carcinoma exhibit a predominant non-hierarchical/haphazard distribution of epithelial cells showing negative and positive nuclear stained cells (IHC intensity 1-3) throughout the thickness of the epithelium.

Finally, regarding intra-observer variability and reliability, there was a positive correlation between the two reading sessions (session 1 and session 2) (r=0.9) **(Figure 8A)**. Similar results were noted for inter-observer reliability, with a positive correlation between the two investigators (r=0.9) **(Figure 8B)**.

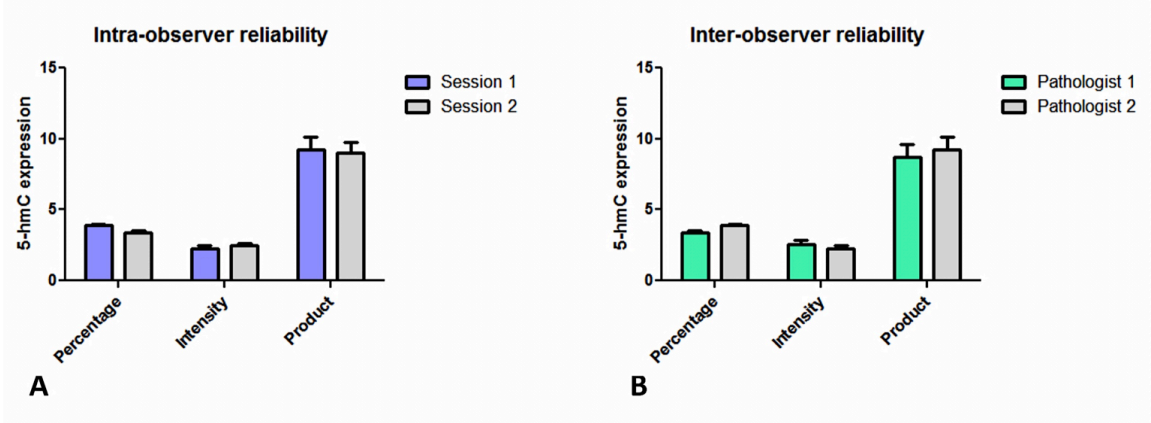


Figure 8. (A) Intra-observer variability and reliability. Comparison of the two reading sessions by one investigator demonstrated a positive correlation between session 1 and session 2 (r=0.9). (B) Inter-observer reliability. Similar results were noted with a positive correlation between the two readings of two investigators (pathologists) (r=0.9).

II. 5-hydroxymethylcytosine expression levels in normal, dysplastic and carcinomatous human keratinocyte lines

These studies were performed to further evaluate and confirm differences in 5-hmC expression between benign human oral epithelial cells and human dysplastic/malignant oral epithelial cells at genomic levels *in vitro*. Cell lines were provided by Dr. Matthew Ramsey, Ph.D. who is the director of the Cell Culture Core Collection in the Brigham and Women's Hospital Department of Dermatology. This core comprises a variety of human well-characterized cell lines including skin and oral keratinocytes, squamous cell carcinoma cells, mesothelial cells and connective tissue fibroblast amongst others. It also provides consultation regarding experimental designs and generation of other cell lines and cultures from other human tissues [66, 67] **(Figure 9)**.

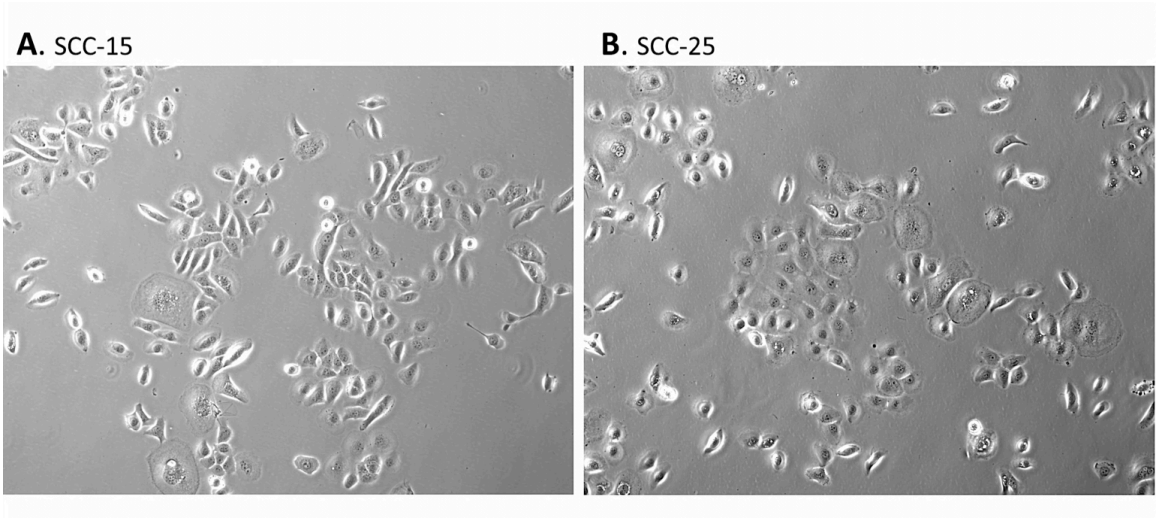


Figure 9. Human carcinomatous oral cell lines. (A) Keratinocytes from human squamous cell carcinoma of base of tongue (SCC-15). (B) Keratinocytes from human squamous cell carcinoma of base of tongue (SCC-25). Both lines are p16- and p53 deficient.

Levels of 5-hmC in all cell lines were analyzed by dot blot. Firstly, all non-inflamed/non-cancerous mortal and immortalized oral keratinocyte lines (OKF4/1.3mMCa, OKF4/TERT-1 and OKF4/E6E7) were compared. The mortal non-inflamed/non-cancerous well-differentiated keratinocyte line (OKF4/1.3mMCa) exhibited the highest levels of 5-hmC, and statistically significant differences were found between 5-hmC expression levels between normal well-differentiated keratinocytes

(OKF4/1.3mMCa) and immortalized normal oral keratinocytes (OKF4/TERT-1 and OKF4/E6E7) **(Figure 10A)**. Then, a non-inflamed/non-cancerous well-differentiated keratinocyte line (OKF4/1.3mMCa) was compared with oral dysplastic mortal and immortalized cell lines (POE9n and POE9n/TERT) as well as one oral squamous cell carcinoma cell line (SCC-71). Dysplastic and carcinomatous cell lines exhibited global decrease of 5-hmC levels when compared to cells representative of normal oral mucosa ($P<0.001$) **(Figure 10B)**.

Further, in a second experiment, a non-inflamed/non-cancerous mortal keratinocyte line (OKF4/1.3mMCa) was compared with both immortalized non-inflamed/non-neoplastic cell lines (OKF4/TERT-1 and OKF4/E6E7) and with several different oral SCC cell lines (SCC-15, SCC-25, SCC-68, SCC-71). Consistent decreases in 5-hmC levels were seen in all carcinomatous cell lines when compared to OKF4/1.3mMCa. However, only the results between the carcinomatous cell lines SCC-15, SCC-68, SCC-71 and OKF4/1.3mMCa were statistically significant ($P<0.001$) **(Figure 10C)**. Finally, all dysplastic and carcinomatous cell lines were compared, and no statistically significant differences were found between those cell lines **(Figure 10D)**.

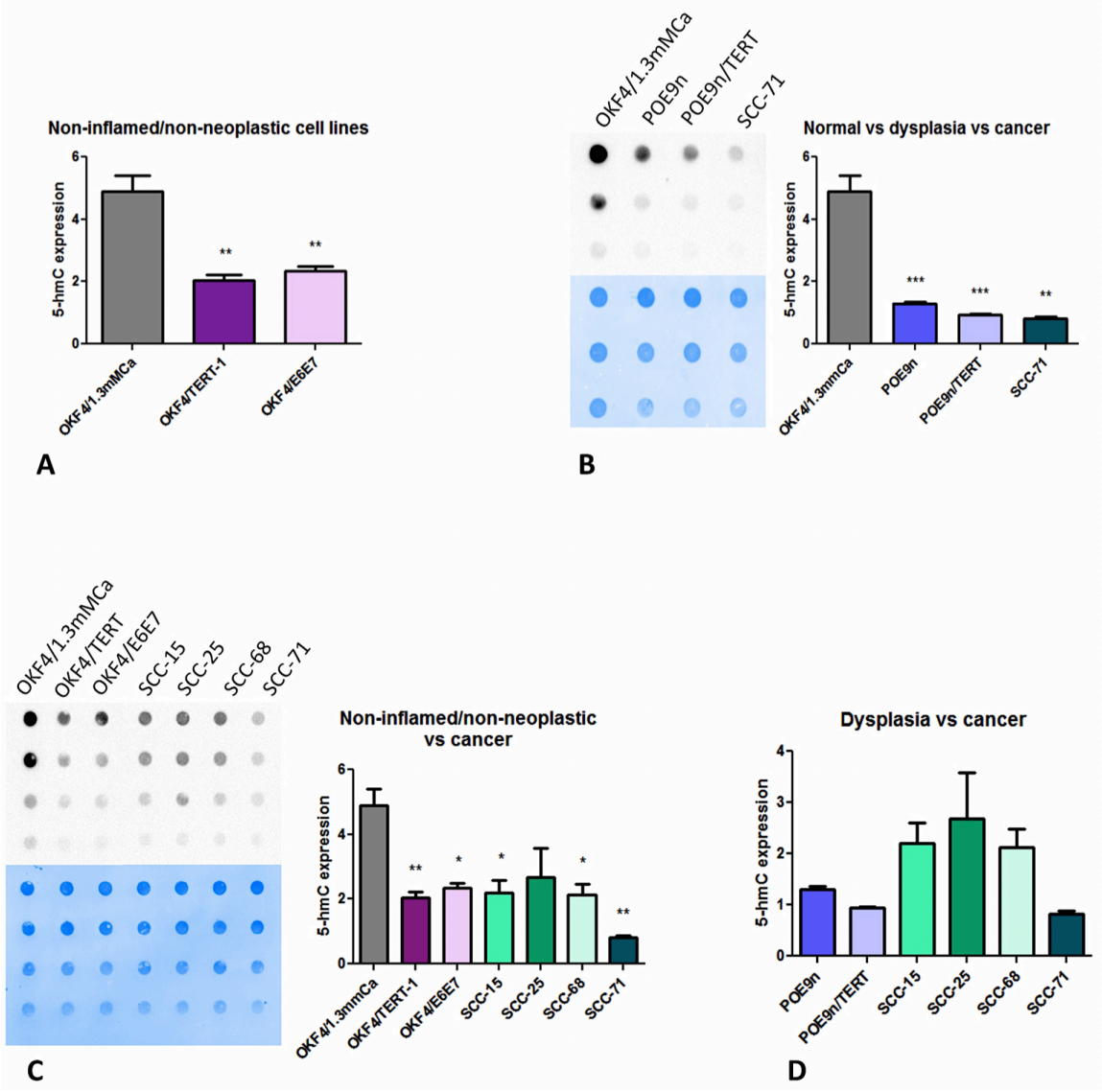


Figure 10. 5-hmC nuclear immunoreactivity in oral mucosal cell lines. (A) ANOVA analysis reveals statistically significant differences in 5-hmC expression between non-inflamed/non-cancerous well-differentiated keratinocyte line (OKF4/1.3mMCa) and immortalized normal oral keratinocyte cell lines (OKF4/TERT-1 and OKF4/E6E7) ($P<0.001$). (B & C) Global 5-hmC expression levels in cell lines by dot blot assay. Methylene blue was used as total genomic DNA loading control. ANOVA analysis reveals statistically significant differences in 5-hmC expression between non-inflamed/non-cancerous well-differentiated keratinocyte line (OKF4/1.3mMCa) and oral dysplastic cell lines (POE9n, POE9n/TERT), as well as with oSCC cell line (SCC-71) ($P<0.001$). Well-differentiated keratinocytes of normal oral mucosa (OKF4/1.3mMCa) retained the strongest nuclear intensity for 5-hmC whereas dysplastic cell lines (POE9n, POE9n/TERT) and oSCC cell line (SCC-71) demonstrated loss. ANOVA analysis also reveals statistically significant differences in 5-hmC expression between non-inflamed/non-cancerous well-differentiated keratinocyte line (OKF4/1.3mMCa) with immortalized non-inflamed/non-neoplastic cell lines (OKF4/TERT-1 and OKF4/E6E7)

and with several different oral SCC cell lines (SCC-15, SCC-68, SCC-71) ($P<0.001$). Consistent decreases in 5-hmC levels were seen in immortalized non-inflamed/non-neoplastic cell lines and all carcinomatous cell lines when compared to OKF4/1.3mMCa. (D) ANOVA analysis reveals no statistically significant differences between all dysplastic and all carcinomatous cell lines.

III. 5-hydroxymethylcytosine immunoreactivity in an established murine model of oral carcinogenesis elicited by 4-nitroquinoline-1-oxide (4NQO)

Based on our results in human tissues in vivo and in vitro, we next sought to determine whether similar findings with respect to 5-hmC expression in oral epithelium could be reproduced in an established animal model of experimentally-induced oral squamous epithelial dysplasia/carcinoma. Animal tissue was received from a collaborative study between the Department of Oral Medicine, Beijing Hospital for Stomatology, School of Stomatology, Capital Medical University, and the Cancer Research Program, North Carolina Central University. Ten animal oral tongue samples (N=10) were received as unstained paraffin embedded tissues on glass slides from an established murine model of oral carcinogenesis elicited by 4-nitroquinoline-1-oxide (4NQO) [64].

Evaluation of IHC staining of animal samples was based on three parameters, as applied to the human specimens described previously. An immunoreactivity score (IRS) ***[a x b = c]*** was derived by multiplying the score representative of the percentage of positive cells ***[a]*** with the number consistent with staining intensity ***[b]***. The resultant product scores ***[c]*** were analyzed statistically among specific foci of interest.

As with normal human oral mucosa, all the cases of normal non-inflamed/non-neoplastic dorsal tongue ($n_T=5$) exhibited diffuse strong nuclear positivity in more than 75% of the cells (mean percentage of positive cells 4.0; mean IHC intensity 3.0). For all the cases examined ($n_T=5$) there was a hierarchical distribution that paralleled maturation in terms of intensity of the staining with the darkest cells (IHC intensity 4) in the top layers of the epithelium. In the stratum spinosum or “middle portion” of the epithelium, which consists of the higher number of layers, the cells uniformly exhibited 5-hmC nuclear immunoreactivity of intensity 3 **(Figure 11A)**.

Cases of dysplasia (n_{OED}=5) and oSCC ($n_{mSCC=}$5) showed intermediate to high percentage of positive nuclear stained cells. For dysplasia, the mean percentage of positive cells was 2.8 and for oSCC the mean percentage of positive cells was 2.6. In terms of the intensity of the staining, all dysplastic (n_{OED}=5) **(Figure 11B)** and oSCC lesions ($n_{mSCC=}$5) exhibited light nuclear immunoreactivity (mean IHC intensity 1.4 and mean IHC intensity 1, respectively) **(Figure 11C)**.

In all dysplastic lesions (n_{OED}=5), the pattern was similar to that seen in our previous results from human OED, where dysplastic cells exhibited light nuclear immunoreactivity (mean IHC intensity 1.4) in the lowest and mid portion of the epithelium, consistent with the presence of dysplastic cells that were confirmed in adjacent, conventionally-stained sections. In 60% of oSCC cases ($n_{mSCC=}$3), tumor islands were composed of cells with light 5-hmC nuclear immunopositivity (mean IHC intensity 1). However, in the other 40% of cases ($n_{mSCC=}$2), tumor islands showed a dichotomy where 85% to 95% of cells exhibited light nuclear immunoreactivity (mean IHC intensity 1) and a subset of cells (about 10 to 15%) demonstrated absence of 5-hmC immunoreactivity (IHC intensity 0).

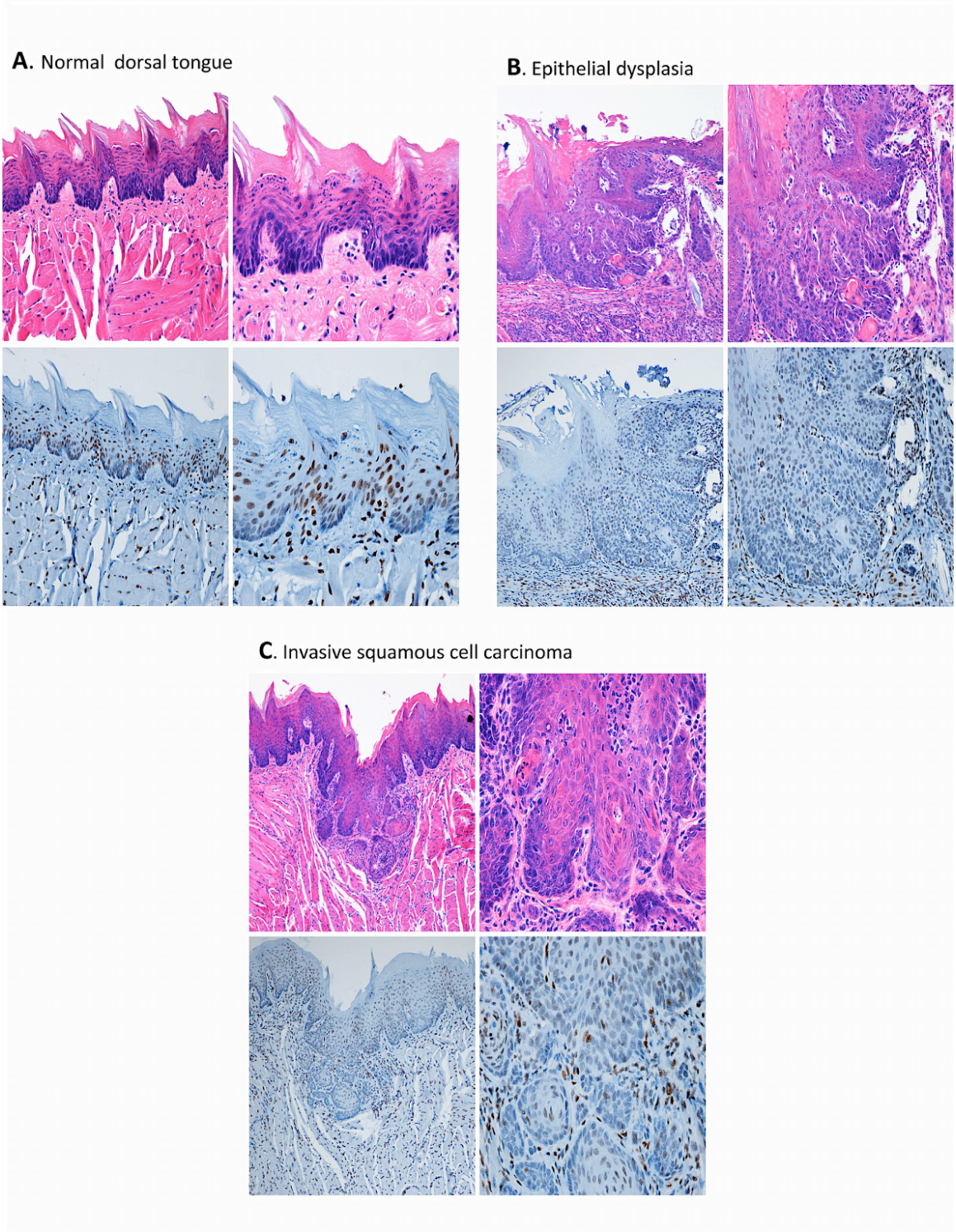


Figure 11. Correlative H&E histology and 5-hmC nuclear immunoreactivity in oral mucosa of a murine model of oral carcinogenesis elicited by 4-nitroquinoline-1-oxide (4NQO). Note preservation of hierarchical pattern of 5-hmC expression in (A) Normal non-inflamed/non-neoplastic dorsal tongue. Dramatic loss of 5-hmC expression in (B) moderate to severe epithelial dysplasia and (C) invasive squamous cell carcinoma (H&E 200, 400X and 5hm-C 200X, 400X).

Regarding the percentage of positive cells and in contrast to findings in human tissues, there were statistically significant differences between 5-hmC immunoreactivity in mucosa of non-inflamed/non-cancerous dorsal tongue compared to dysplasia and oral squamous cell carcinomas ($p<0.001$) **(Figure 12A)**. In addition, there was a statistically significant difference with regard to the intensity score of benign dorsal tongue, in contrast to dysplasia and oral squamous cell carcinoma lesions ($p<0.001$) **(Figure 12B)**. When the product or 5-hmC immunoreactivity score (IRS) was evaluated between murine dysplastic and carcinomatous lesions compared to normal mucosa there was a statistically significant result ($p<0.001$) **(Figure 12C)**.

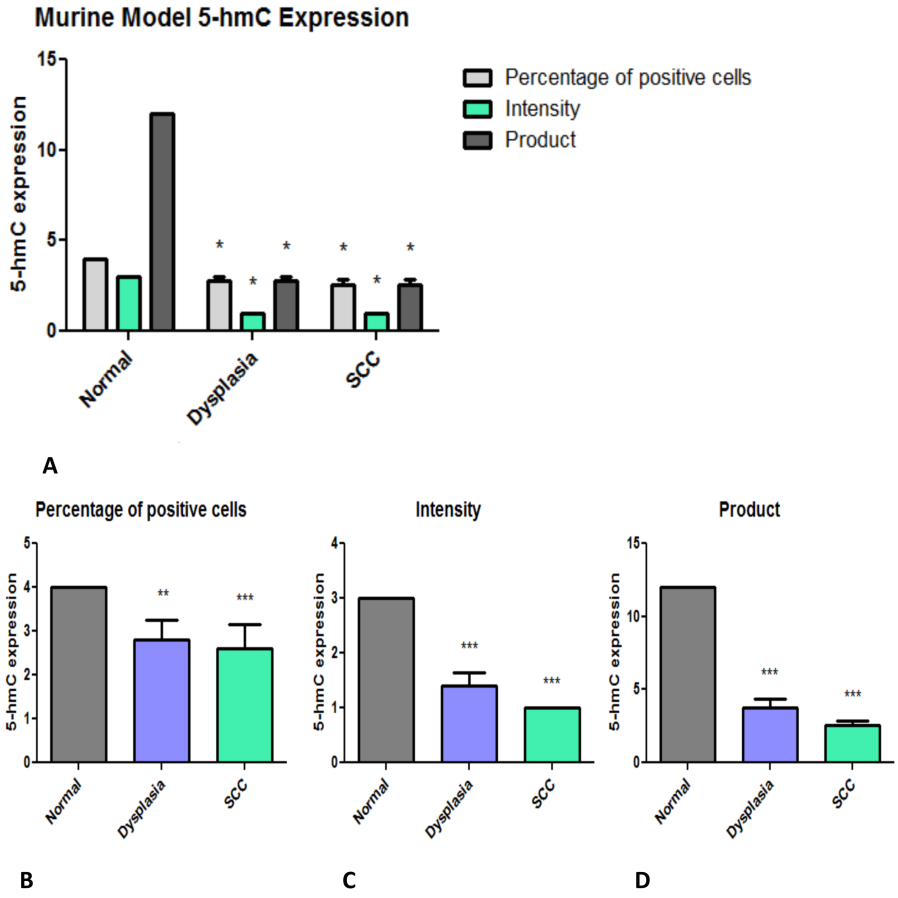


Figure 12. (A, B & C) ANOVA analysis reveals statistically significant differences in 5-hmC immunostaining with respect to percentage of positive cells, nuclear staining intensity and resultant (IRS) product score ($p<0.001$). Normal non-inflamed/non-neoplastic dorsal tongue retained the strongest nuclear intensity for 5-hmC whereas dysplasia and oSCC demonstrated loss.

DISCUSSION

In this study, we have i) confirmed that the epigenetic marker 5-hmC, is differentially expressed in patient biopsies of oral normal and reactive oral mucosa when compared to squamous mucosal dysplasia and carcinoma; ii) demonstrated that alterations in 5-hmC expression that typify oral squamous dysplasia and carcinoma are also detectable *in vitro* using human cell lines from normal and carcinomatous sources; and iii) characterized similar alterations in 5-hmC in an *in vivo* animal model of oral dysplasia/carcinoma, now facilitating further mechanistic studies to address a potential functional role of 5-hmC loss in oral oncogenesis.

Loss of 5-hmC as an epigenetic biomarker for oral dysplasia

Our results suggest that loss of expression of 5-hmC could serve as a potential biomarker for the characterization and possible diagnostic recognition of oral dysplastic/premalignant lesions, as well as oral squamous cell carcinoma. Specifically, we have confirmed that there is a loss of this epigenetic biomolecule in dysplastic oral pre-malignant lesions and oSCC, in contrast to its presence in non-cancerous lesions. To our knowledge, this is the first study that also reports the comparative expression levels and patterns of 5-hmC in normal, inflamed and dysplastic oral mucosal lesions. Importantly, we have shown that 5-hmC is retained in reactive lesions such as frictional keratosis and inflammatory (immune mediated lesions) such as lichen planus. Because these lesions are often confused or are perhaps difficult to separate from dysplastic premalignant oral lesions, biomarkers are needed to facilitate differentiation in difficult cases. In this manner, this project illustrates the potential utility of 5-hmC biomarker in discriminating between benign reactive lesions and dysplastic lesions at the time of biopsy. Additionally, immunohistochemical staining for 5-hmC could be useful in clarifying which leukoplakias (potentially malignant lesions) will likely progress to oral squamous cell carcinomas. Because we correlated loss of 5-hmC with histopathology of lesions that could be easily diagnosed by routine H&E staining, it could be argued that it remains unclear as to whether loss of 5-hmC will serve as an accurate gauge of early

oncogenesis in difficult and borderline lesions where follow-up to confirm biological potential is not practical. Therefore, our approach was also to confirm the biological fidelity of loss of 5-hmC both *in vitro* in relevant cell lines and in an animal model of oral epithelial carcinogenesis.

Interestingly, our results both support the findings reported by *Haffner et al*, [47] as well as extend them in terms of providing insight into understanding this epigenetic event. Their group recently showed that there is a hierarchical pattern of expression of 5-hmC in normal murine oral dorsal tongue, as well in other human epithelia. The highest 5-hmC levels were observed in the nuclei of terminally differentiated cells (top layers of the epithelium), while the basal and suprabasal cells showed low 5-hmC nuclear levels. These results implied that the expression of 5-hmC may correlate with the stage of differentiation of the squamous epithelial cells, with the highest expression in well-differentiated cells, and the basal cells or immature cells showing low levels of expression. Our data with regard to normal human non-inflamed/non-neoplastic tissue demonstrated precisely this pattern of staining, and this pattern was also present in murine oral mucosa.

However, we also found that changes such as a keratinization and inflammation may alter the expression patterns of 5-hmC. For instance, in benign frictional keratotic lesions that exhibit hyperkeratinization (thickened layer of orthokeratin), we did not detect a hierarchical pattern of expression. Rather, in these lesions we noted three characteristics of the pattern of staining: first, the positively stained cells were arranged in a haphazard distribution (non-hierarchical) with the majority of the cells showing high levels of 5-hmC. Secondly, we noticed that a small percentage of cells (less than 10%) in the stratum spinosum and top layers of the epithelium exhibited light to no nuclear staining, implying low or absent levels of 5-hmC similar to what is seen in basal cells. Finally, in the basal cell layer, in contrast to normal non-inflamed/non-neoplastic mucosa, cells showed a dichotomy of staining, with both light and dark nuclear reactivity (IHC intensity 1, 2 or 3). One possible explanation for this is that external stimuli or injury may alter and perturb maturation patterns, with cells with variable differentiation properties in different layers of the epithelium. While some cells may retain replicative potential, others may exhibit accelerated differentiation stages. In such a situation we might anticipate a disorganized pattern with mature cells in the lower third of the epithelium

and more immature, perhaps progenitor or stem-like cells with replicative potential, present in the upper layers. Additional studies correlating 5-hmC reactivity with markers of squamous epithelial differentiation (e.g. various cytokeratins, involucrin, filaggrin, etc.) will be required to further test this hypothesis. Currently, how the process of homeostasis is maintained in the oral epithelium, as well as how it responds to external stimuli and injuries has not been yet clearly understood [68].

The oral epithelium consists of stratified squamous epithelium. In the gingiva, hard palate and dorsal tongue there is keratinizing epithelium similar to that one seen in skin, although in mucosa, the characteristic basket-weave stratum corneum of a 'dry' squamous epithelium is lacking. Histologically, the oral epithelium is divided from the bottom (in contact with the connective tissue) to the top in zones termed the basal, spinous, granular and corneal layers. The progenitor cells are thought to be present in a thin basal cell layer in areas such as floor of mouth, but can also be present in the lower two to three layers in areas where there is thicker epithelium, like the buccal mucosa [68]. Theories explaining epithelial stem cell biology have been recently revisited with the appearance of more sophisticated tools and mathematical modeling, leading to new paradigms that explain the cellular and molecular events in the differentiation of oral epithelium based on two models, the invariant asymmetry model, and the neutral drift model [69].

The epidermal proliferative unit model, first described by Potten in 1974, proposed that the organization of cells in the epithelium is based on an invariant asymmetry. One epidermal proliferative unit is composed of one central, slowly dividing stem cell that generates peripherally more rapidly dividing cells called transient amplifying cells (TA) The stem cell located in the basal cell layer will always divide symmetrically to give rise to two TA cells. Subsequently, these TA cells will generate new TA cells as well as post-mitotic differentiated cells within the epithelium that will senesce or terminally differentiate after multiple divisions. The model illustrates that there are stem cells within the basal layer that invariably divide symmetrically to generate a heterogeneous yet heirarchical mixture of suprabasal TA cells and differentiated cells [70, 71]. Accordingly, each stem cell gives rise to TA cells and then differentiating cells that occupy a single clone or epidermal proliferative unit.

The more recently studied model for stem cells in the epithelium is the neutral

drift model. This has been applied so far for skin, esophagus, intestinal crypt and testes. In contrast to the invariant asymmetry model, one or more stem cell/progenitor populations may be present in the basal layer with cell divisions being either asymmetrical (yielding a new stem cell and a TA cell), or symmetrical (yielding either two new stem cells, or two TA cells). This, each new clone of cells may be associated with a variable number of stem cells, depending on variations in the division sequence [72-74].

Clearly, when squamous mucosal homeostasis is perturbed, stem cell populations may be stimulated into a number of aberrant division pathways. 5-hmC has recently been shown to be a biomarker for melanoma aggressiveness [58], and this attribute may reside in its association with more primitive, stem-like cancer cells that mediate virulence. Indeed, 5-hmC plays a well-recognized role in plasticity and fate determination during embryogenesis, and thus its relationship to more or less differentiated cell populations in oral squamous mucosal lesions should not be surprising. We speculate that external stimuli that may stimulate deviation from a proliferative and differentiation program that normally supports hierarchical cell transitions may alter the kinetics of stem cell division, thus resulting in a shift from a normal state of invariant asymmetry to one more in keeping with neutral drift. Such an event might explain the juxtaposition of 5-hmC-negative and 5-hmC-positive cells in the mid-epithelial strata of benign proliferative lesions. Thus, correlation of 5-hmC expression with biomarkers of cell replication and stemness may represent a valuable future avenue for investigation.

There have been multiple attempts to identify specific biomarkers that will characterize stem cells in many tissues. In the oral tissues most studies have focused in identifying cancer stem cells in oral premalignant lesions like oral dysplasia and oral squamous cell carcinomas, and little has been done for the identification of these cells in normal oral epithelium. A few of the biomarkers that characterized oral mucosal stem cells include CD44H, CD71; a number of keratins (CK) such as CK15, CK19; Nestin, Oct3/4, Nanog, Sox 2 and ABCG2 amongst others [69]. CD44H is a Type 1 transmembrane glycoprotein involved in cell–cell interactions, cell adhesion, and migration. It has been studied in human gingiva [75]. CD71 is a transferrin receptor highly expressed in actively cycling cells with lower expression in slower cycling keratinocyte stem cell, and it has also been studied in human gingiva [76]. CK15 is an

intermediate filament protein expressed in human hard palatal mucosa and hair follicle bulge stem cells and less differentiated keratinocytes in neonatal mice [77, 78]. Likewise, this cytokeratin has been identified in association with a subpopulation of squamous mucosal epithelial cells that reside at the tips of rete-like downgrowths of the dorsal tongue of the mouse [79]. In addition to being slow-cycling, these cells are also characteristically apoptosis-resistant [80], although in certain inflammatory conditions, activation of members of the p53 family may induce cell death [81]. CK19 is another intermediate filament protein expressed in human gingiva, hard palatal mucosa and glabrous skin stem cells. Nestin is a Class VI intermediate filament expressed in developing neuroepithelial stem cells and human gingival stem cells [74, 78]. Oct3/4, Nanog and SOX2 are transcription factors that influence self-renewal in stem cells. Oct3/4 and Nanog have been studied in human gingiva, whereas SOX2 has been studied in murine tongue [82-85]. Finally ABCG2 is a cell membrane transporter which function is to pump a wide variety of compounds out of cells, and has been found in stem cells from several different tissues including human derived buccal mucosa cultured cells [86, 87].

In future studies, any of these biomarkers could be utilized to further characterize those lightly stained cells in our cohort, thus providing more information with regard to its biological functions and will perhaps answer the question as to whether these cells are stem cells.

With respect to lichen planus, we found similar levels of 5-hmC expression comparable to non-inflammatory reactive conditions such as frictional keratosis, and to normal mucosa at the edges of fibromas. In the majority of our samples there was no hierarchical pattern of 5-hmC expression. There is scant information regarding epigenetics of oral inflammatory diseases [88]. Nevertheless, epigenetics of other systemic autoimmune diseases have been well documented. As mentioned earlier in this manuscript, DNA methylation is the most common studied epigenetic mechanism. It has been established that some systemic autoimmune or immune-mediated diseases exhibit either hypermethylation or hypomethylation of DNA. With regard to oral lichen planus, a few studies have reported results implying that hypermethylation of DNA is implicated in its development, although further investigations are needed. *Fonseca-Silva et al.* reported that there was increased protein expression of DNA methyltranferases (DNMT3B and

DNMT1) in oral lichen planus samples compared to control oral mucosa. As was previously mentioned, DNT enzymes catalyze the addition of a methyl group to DNA. Hence they hypothesize that increased expression of these enzymes can influence the development of oral lichen planus through hypermethylation of some particular genes [86,87]. In our study we did not find statically significant differences between the levels of 5-hmC in normal mucosa at the edges of fibromas or frictional keratotic lesions, yet we identify higher levels of this epigenetic mark when compared to dysplasia and oral squamous cell carcinoma. This may suggest that the loss of 5-hmC in premalignant and malignant lesions is not due to inflammation per se, and perhaps supports the fact that this epigenetic mark could serve as a biomarker that will distinguish between inflammatory and neoplastic diseases. However, to prove the fact that hypermethylation is implicated in lichen planus, specific genes should be identified and evaluated with regard to their methylation status.

Loss of 5-hmC is also present in vitro in cultured cells obtained from oral dysplasia and oral squamous cell carcinoma

With regard to the results concerning the cell lines, we noticed similar findings to those seen in human tissues and the animal model, where there was a global decrease in the levels of 5-hmC in oral dysplastic cell lines and oral squamous cell carcinomas compared to cell lines of normal mucosa. Interestingly, we found statistically significant results when these pre-malignant and carcinomatous cell lines were compared with the normal mucosal cell lines that were cultured using a high concentration of calcium (OKF4/1.3mMCa), which represented more differentiated keratinocytes typical of in situ findings. Thus we consider this cell line to be the most appropriate one when comparing normal mucosa versus dysplastic and cancerous oral lesions.

It has been shown that calcium is important for regulation of epithelial differentiation *in vitro.* Nevertheless, the function of calcium in keratinocytes *in vivo* is yet to be determined. It has been reported that high concentration of calcium is required for keratinocyte stratification and desmosome assembly *in vitro* [89]. When keratinocytes from skin are grown in a medium with low calcium concentration, the cells continue to proliferate and lack desmosomes. However, when the amount of calcium is increased at

concentrations higher than 0.1 mM, they usually express differentiation markers such as CK1, CK10 as well as other cytoplasmic proteins seen in more differentiated cells of the epithelium such as loricrin and profilaggrin often found in the stratum corneum [90, 91]. Therefore in cell cultures, to produce cornified envelopes (seen in well-differentiated keratinocytes of the skin, and that consist of a combination of keratins tightly packed with an insoluble group of proteins that are cross-linked by transgutaminases and surrounded by a lipid layer [92]) high calcium conditions are needed [93]. Importantly, it has also been established that *in vivo* there is a calcium gradient in the skin that increases from the basal cell layer to the granular and top layers of the epithelium [94], supporting the fact that well-differentiated keratinocytes have greater amounts of calcium when compared to basal cells or immature keratinocytes.

Moreover, we noted that immortalized keratinocyte lines (OKF4/TERT-1 and OKF4/E6E7) have lower levels of 5-hmC, and these results were statistically significant when compared to normal mortal keratinocyte lines (OKF4/1.3mMCa). Of note, these immortal cell lines express pathways capable of evading senescence, and hence have high replicative potential; they are considered "replicatively immortal". However, they exhibit normal growth characteristics and differentiation potential. Accordingly, these are not considered neoplastic cell lines. Nevertheless, it has been reported that because they have the ability to induce growth arrest and have unlimited replicative potential this could predispose them to further modifications and malignant transformation [60, 95].

Our results with the cell lines further support a negative correlation between the expression of 5-hmC and cells with high replicative potential. This perhaps is also supported by the fact that dysplastic cells and cancer cells in our study collectively exhibit low levels of this epigenetic mark when compared to the normal counterpart. It is well known that cancer cells, likewise, have high replicative potential, although they are also characterized by other features that make them malignant: lack of normal differentiation potential, capacity to metastasize and invade tissues, induction of angiogenesis, amongst others. Consequently, it has yet to be determined if loss of 5-hydroxymethylcytosine in these cell lines is a result of modifications in the senescence mechanisms of a cell, or results from a combination of modifications in senescence mechanisms with other pathways pertinent to dysplastic/pre-malignant and neoplastic cells.

Establishment of an animal model for further study of loss of 5-hmC in oral dysplasia/squamous cell carcinoma

While our results in patient tissues and human cell lines are provocative with respect to the potential utility of detecting loss of 5-hmC as an adjunct to the diagnosis of certain oral pre-malignant and malignant lesions, we have not provided significant insight into how 5-hmC loss may play a mechanistic role in oncogenesis. The recent work by *Lian et al* [58] in melanoma models raises the possibility that reconstitution of the TET2-mediated 5-hmC landscape in cancer may be an important new direction for epigenetic therapy. If so, loss of 5-hmC would be implicated causally in the oncogenic sequence. Indeed, *Lian et al*. have documented the association of loss of 5-hmC with more than 2000 virulence-conferring genes in melanoma, implying that altered hydroxymethylation may tip the scale in the direction of the aggressive and unregulated growth potential that typifies cancer cells. In order to explore such possibilities in oral dysplasia and squamous cell carcinoma, *in vivo* models will be required. Accordingly, in this study we also investigated oral dysplasia and cancer with respect to a well-established model of oral carcinogenesis.

Our findings showed remarkable similarities between the mouse model under study and human disease. However, we did not evaluate comparatively induced inflammatory and hyperplastic lesions in the murine model, and this will need to be performed before definitive conclusions may be reached regarding the potential utility of this approach. Nonetheless, the ability to induce experimental oral dysplasia and carcinoma in a mouse model in which 5-hmC is lost now provides a valuable opportunity to evaluate the effects of TET2-mediated reconstitution of 5-hmC on the genesis and progression of oral dysplasia/carcinoma in a model system relevant to human disease. Available agents (e.g. DNA methyltransferases as well as other agents under development to modify TET2 expression will aid in bringing these important new experimental approaches to the forefront.)

CONCLUSION

Screening for potentially malignant oral lesions is typically confounded by difficulty in discriminating between those that are reactive/inflammatory versus those that are premalignant in nature. There is an urgent need for the discovery of specific biomarkers and related molecular events that would have optimal predictive capacity for the identification of those dysplastic lesions that most likely will progress to oral squamous cell carcinoma over time. Epigenetic alterations have been reported to play a crucial role in the development of several types of cancer. Specifically, DNA methylation and demethylation regulate many biological processes that are fundamental to the genesis of cancer. Loss of 5-hydroxymehylcytosine has been found to be an epigenetic event in many cancers, including in its recent implication oSCC. In this study, 5-hmC distinguishes oral dysplasia and oral squamous cell carcinoma from benign and reactive inflammatory lesions with high sensitivity and specificity. Moreover, our results with respect to oral keratinocyte cell lines supported the use of 5-hmC biomarker and also provide new data with respect to the use of cell cultures for the evaluation of epigenetic events related to oral pathology.

Finally, the application of a well-established animal model for the induction of oral dysplasia/carcinoma to studies that verify the phylogenetic preservation of loss of 5-hmC in oral cancer establishes and additional avenue for elucidation of epigenetic pathways in oral oncogenesis. In conclusion, our research directed at evaluating the expression and functional characterization of 5-hmC in normal and inflamed oral mucosa, oral premalignant lesions and oral squamous cell carcinoma, suggests a potential new strategy for the diagnosis of premalignant lesions of the oral cavity. Furthermore, our work could have an impact in therapeutic approaches for oral squamous cell carcinoma contributing to the field of personalized medicine, which is the goal of this generation's work towards the management of cancer.

SUGGESTIONS FOR FUTURE PROJECTS

Epigenetic alterations in oral lesions, particularly in premalignant lesions such as oral epithelial dysplasia, as well as oral squamous cell carcinoma have just recently been studied. Hence there is still much to do and there seem to be tremendous opportunities to explored in this field that will aid in the understanding of this neoplastic process. In addition to some of the recommendations we presented in the discussion, the diagnostic utility of the biomarker 5-hydroxymethylcytosine could be further explored and support through various other experiments. Next generation sequencing technologies could be employed to define occurrence and frequency of mutations in genes encoding epigenetic regulators, such as genes encoding DNA methylation/demethylation enzymes, histone modifying proteins and chromatin remodeling proteins. Furthermore, we recommend the evaluation of 5-hmC in genes specifically associated with the development of oral squamous cell carcinoma such as Tp53, CDKN2A, HRAS and PIK3CA, to further prove if hypomethylation or hypermethylation occurs in carcinomas.

Moreover, the expression of enzymes that regulate methylation and demethylation processes, such as the DNA methyltranferases and the ten-eleven translocation family of 5-mC hydroxylases (specifically TET 2, which mutational inactivation have been reported to be present in other malignancies such as myeloid leukemia and melanoma) could be investigated.

This work will provide additional information with regards to the global methylation state of DNA in these oral lesions, and will aid to our understanding of the epigenetic mechanisms associated with the development of oral neoplasms.

With regard to the utilization of oral keratinocyte lines, there is much to explore that will validate the use of 5-hmC as a biomarker for the identification of premalignant and malignant conditions of the oral cavity. The evaluation of the growth rate of the cells in the different cell lines and its association with the expression of epigenetic markers will serve to clarify the low expression of this biomarker in some of the cell lines form normal keratinocytes.

Furthermore, since human papilloma virus (HPV) associated oral squamous cell carcinomas behave differently from those that are not induced by the virus, it will be interesting to perform these experiments in samples from patients positive for HPV as

well as in animal models were the cancers are induced by viral proteins. Along the same lines the presence of human papilloma virus in the cell lines that we used for this study has not been evaluated; hence we recommend that further assessment to discover if this epigenetic mechanism is similar or different between the two types of neoplasms.

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