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In-Silico Analysis of Chromatin Modifiers and Profiling of Histone Deacetylases (HDAC's) in Human Oral Cancer

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Histone modifications have been demonstrated to play a significant role in oral squamous cell carcinoma (OSCC) epigenetic regulation. An *in-silico* analysis of The Cancer Genome Atlas (TCGA) of various histone acetyl transferases (HATs) and histone deacetylases (HDACs) suggested that HATs do not differ between normal and tumor samples whereas HDAC2 and HDAC1 change maximally and marginally respectively between normal and tumor patients with no change being noted in HDAC6 expression. Hence, this investigation was carried out to validate the expression states of HDAC 1, 2 and 6 mRNAs in buccal mucosa and tongue SCC samples in an Indian cohort. Buccal mucosa and tongue squamous cell carcinoma tissues with intact histopathology were processed for RNA isolation followed by cDNA synthesis which was then subjected to q-PCR for HDACs. The average RNA yield of the tongue tissue sample was ~2 μg/mg of tissue and the $A_{260/280}$ ratios were between 2.03 and 2.06. The average RNA yield of buccal mucosa tissue sample was ~1 μg/mg of tissue and the $A_{260/280}$ ratio were between 2.00 and 2.08. We have demonstrated that HDAC2 was overexpressed in tongue and buccal mucosa samples. Over-expression of HDAC2 imply potential use of HDACi along with standard chemotherapeutic drug in oral cancer treatment.

Key Words: *Oral Squamous Cell Carcinoma; Histone Acetylases; Histone Deacetylases*

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INTRODUCTION

Oral cancer is one of the most common cancers in the world with oral squamous cell carcinoma (OSCC) encompassing 90~95% of its subtype.[1] It is the most common cancer occurring in men in India and accounts for 16.1% of all cancers occurring in this group. Oral cancer is the second most common cancer in India and comprises 10.4% of all cancers recorded in the country.[2] OSCC is a debilitating malignancy with aggressive phenotypes and behavior leading to poor prognosis and limited therapeutic options in advanced cases. Genetic and epigenetic alterations of oncogenes and tumour suppressor genes have been implicated during the various steps of carcinogenesis of OSCC. Though genetic modifications during development of OSCC have been well elucidated, epigenetic alterations for OSCC have been inadequately described in the literature.[3]

Histones are key structural proteins which constitute the nucleosome that are critical for DNA assembly into chromatin.[4] There are 5 types of histones, H2A, H2B, H3 and H4 which constitute the core proteins and H1 histone which is the linker histone. The N-terminal regions of histone proteins are substrates for a number of enzymes that affect post-translational modifications of these proteins. Histone modifications have been demonstrated to play a significant role in OSCC epigenetic regulation.[5-7] Amongst these modifications; H3 hypo/hyperacetylation, H4 hypoacetylation and H3 methylation reduction/increase have shown notable alterations.[7,8]

Broadly, histone acetylation is regulated by two distinct classes of enzymes – histone acetyl transferases (HATs) and histone deacetylases (HDACs). HATs transfer the ace-

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tyl moiety from acetyl coenzyme A to specific lysines on histones while HDACs as a group of enzymes to catalyze the removal of acetyl group/s from the histone lysine residues producing their effect.

Three major families of HATs have been identified in humans – the Gnc5-related *N*-acetyltransferase family (GNAT), the MYST family (MOZ, Ybf2, Sas2, TIP60) and the orphan family (CBP/ EP300 and nuclear receptors).[5] A number of studies have implicated HATs as both oncogenes and tumour suppressors - alterations in HAT levels often occur without DNA mutations in cancers.[9-11] Amongst these HATs, the global transcriptional coactivator p300 has been observed to have altered expression in some tumours – their somatic mutations being identified in multiple cancers with the resulting loss of heterozygosity implicating them as tumor suppressor.[12] HATs such as p300 have also been reported to induce hyperacetylation in OSCC due to overexpression and hyperacteylation of p300.[13] As such, there is a paucity of data on the role on HATs in the progression of OSCC.

HDACs have been classified into four categories depending upon their sequence homology to yeast original enzyme and domain organization. A total of 18 HDAC subtypes have been identified in mammalian cells so far.[14] Class I, II, and IV comprise the classical Zn+-dependent HADCs while Class III is composed of NAD+-dependent sirtuin.[15,16] Class I HDACs are comprised of HDAC1, 2, 3, 8; Class II HDACs are sub-categorized into Class IIA (HDAC4, 5, 7, 9) and Class IIB (HDAC6 and 10) while Class IV HDACs only contain HDAC11 which is known to have features similar to Class I and Class II HDACs.[14] Class III HDACs contain seven sirtuin proteins (SIRT1-7) that do not share homology with other classes of HDACs.

HDACs have been extensively scrutinized for their roles as epigenetic modifiers in multiple cancers. Enhanced HDAC activity in human tumours has been shown to lead to conformational changes within the nucleosome, which have resulted in transcriptional repression of the genes involved in differentiation and negative regulation of cell proliferation, migration and metastasis.[17,18] However, studies on their significance in OSCC, particularly tongue and buccal mucosa, are scarce in the literature.

HDAC1[19], HDAC2[19,20], HDAC6[21], HDAC8[22] and HDAC9[23] have been reported to be upregulated in OSCC and associated with advanced stages and poor prognosis.[20] Likewise, SIRT1 expression has been found to be elevated in OSCC cells.[24] These limited findings suggest that altered HDAC expression can have varying effects on carcinogenesis of OSCC. Furthermore, the role of individual HDACs on buccal mucosa squamous cell carcinoma (SCC) and tongue SCC is equivocal.

The Cancer Genome Atlas (TCGA) project, a multi-institutional consortium, has generated comprehensive molecular profiling of key genomic and transcriptomic changes across 33 types of cancer.[25] It uses genome, transcriptome and proteome sequencing along with clinical data from 11000 patients. Head and neck squamous cell carcinoma (HNSCC) sample collection from TCGA contains 528 cases for RNA sequencing. An *in-silico* analysis of TCGA of various HATs and HDACs suggested that the expression of only some of the HDACs were altered in multiple HNSCCs. HDAC2 and HDAC1 demonstrated maximum and marginal differences respectively while HDAC6 showed no differences between normal and tumour patients. Hence, this investigation was carried out to measure and validate the expression states of HDACs 1, 2 and 6 of mRNA levels in buccal mucosa and tongue SCC in an Indian population sample.

MATERIALS AND METHODS

1. TCGA data analysis

The RNA seq profiling data sets of HNSCC patients were downloaded from TCGA data portal. A total of 523 anonymized patients in the TCGA database were identified as having primary HNSCC. All HATs and HDACs were analyzed using TCGA data for gene expression. Graphical heatmap representations were constructed with heatmap3 package of R statistical software.

2. Sample collection

Buccal mucosa and tongue tumour samples were retrieved from the Tumour Tissue Repository (TTR) at Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Navi Mumbai, India. The project was approved by Institute's human ethics committee vide #164 dated 27/04/2015. The tissues had been stored at −80℃ for varying period of time. The diagnosis, classification of tumor type, TNM status and the date of surgery were obtained from the Electronic Medical Record (EMR) of Tata Memorial Centre (TMC), Navi Mumbai, India. The tissue samples were naive and were free from any biological hazard (Table 1).

3. Haemotoxylin and Eosin staining

Tissue sections (~4 μm) were stained with Haemotoxylin and Eosin (H&E) to visualize the cells. The sections were stained with haemotoxylin by incubating them for 2 minutes. This was followed by 2 washes under running tap water for 5 minutes each. The sections were dehydrated using 70% and 95% alcohol for 10 minutes sequentially. The slides were further counter-stained with Eosin Y for 1 minute followed by two washes with 100% alcohol. Subsequently, the residue was cleared in two changes of xylene for five minutes each. Finally, slides were mounted with DPX mountant (xylene-based) and dried at 37℃ for 1 hour. The stained slides were examined under the microscope (10X, 20X and 40X) for gross structure, tumour content and nuclear cytoplasmic ratios. Tissue samples with more than 70% tumour content were processed further and included in the study.

4. RNA isolation

Total RNA isolation was done for all the tissue samples. Tumour specimens were finely minced, homogenized and RNA was extracted using the RNA miniprep kit (Agilent)

TABLE 1. Human tissue samples obtained from buccal mucosa and tongue

Sample	Age at the time of surgery (years)	Histopathology	Tumour content
BM 0001	27	Well differentiated SCC	80%
BM 0002	54	Moderately differentiated SCC	90%
BM 0003	55	Moderately differentiated SCC	70%
BM 0004	40	Poorly differentiated SCC	70%
Ton 0001	57	Poorly differentiated SCC	80%
Ton 0002	44	Moderately differentiated SCC	90%
Ton 0003	51	Moderately differentiated SCC	80%
Ton 0004	60	Well differentiated SCC	70%

TABLE 2. List of primers used for RT-PCR

Genes	Forward primer	Reverse primer
HDAC1	ATATCGTCTTGGCCATCCTG	TGAAGCAACCTAACCGATCC
HDAC2	GGGAATACTTTCCTGGCACA	ACGGATTGTGTAGCCACCTC
HDAC6	AGTGGCCGCATTATCCTTATCC	ATCTGCGATGGACTTGGATGG

as per manufacturer's instruction. RNA absorbance was recorded by Nanodrop (NanoDrop 2000c spectrophotometer) at A230, 260 and 280 nm.

5. RNA gel electrophoresis

RNA (2 μg) was taken from the stock; dye was added and run on a denaturing agarose gel to check for the quality of RNA. After the gel electrophoreses, the gel is then stained in ethidium bromide (0.5 μg/mL) containing water, visualized on the UV light box and photographed.

6. cDNA synthesis

DNase1 treated RNA (~1 μg) was used for cDNA synthesis according to the manufacturers' instructions (cat. K1632). The gene specific primers were designed for RPS13 - Forward primer:GCTCTCCTTTCGTTGCCTGA, Reverse primer: ACTTCAACCAAGTGGGGACG. The generated cDNA was used for real-time PCR reaction using QuantStudio 12KFlex. Amplification plot and melt curve were analyzed to check the Ct values. Heatmap representations were constructed to identify the expression of HDACs using R 3.6.Primers used for HDACs have been mentioned in Table 2.

RESULTS

1. *In-silico* analysis of HDACs in HNSCC samples

Analysis of the RNA-seq data in HNSCC samples showed that HDAC2 is highly expressed in tumor samples compared to normal samples (p=0.0002). HDAC1 is marginally upregulated (p=0.05) in tumor samples compared to normal samples (p=0.05) while HDAC6 exhibits no change between normal and tumor samples (Fig. 1). Analysis of HATs showed that they do not differ between normal and tumor samples (Fig. 2).

2. Histopathological analysis of HNSCC samples

The histopathological analysis of the buccal mucosa SCC samples revealed that the lesions were poorly circumscribed. On higher magnification, the lesion could be seen to be made up of hyperchromatic cells. A number of mitotic figures were identified and cluster of necrotic cells were noted in different sections of the slide suggesting that these regions are devoid of blood supply. Pleomorphic cells of various shapes could be identified (Fig. 3A).

The histopathological analysis of tongue SCC samples showed an increase in fibrotic tissue content with an infiltration of group of cells clustered together. These nests of cells contained dysplastic cells with increased nuclear-cytoplasmic ratio, cells with abnormal mitotic activity, hyperchromatic nuclei, and loss of polarity. The cells attempted to form glandular structures but were highly distorted. Ill-defined cell borders, finely granular nuclear chromatin was present along with the characteristic keratin whorls (Fig. 3B).

3. RNA and cDNA quality of HNSCC samples

The average RNA yield of the tongue tissue sample was ~2 μg/mg of tissue and the $A_{260/280}$ ratio was between 2.03 and 2.06. The average RNA yield of buccal mucosa tissue sample was ~1 μg/mg of tissue and the $A_{260/280}$ ratio was between 2.00 and 2.08 (Table 3). Two distinct bands could be seen on the agarose gel corresponding to 28S and 18S ribosomal RNA (Fig. 4). Real time PCR was carried out to find Ct values of buccal mucosa and tongue samples. The value for RPS13 ranges between 18 to 24 suggesting that the quality of cDNA was good (Table 4).

4. HDAC2 was differentially expressed in HNSCC samples

Real time PCR was carried out for HDAC1, HDAC2 and HDAC6 in tongue and buccal mucosa tumor samples. Heatmap analysis for mRNA expression of HDACs in

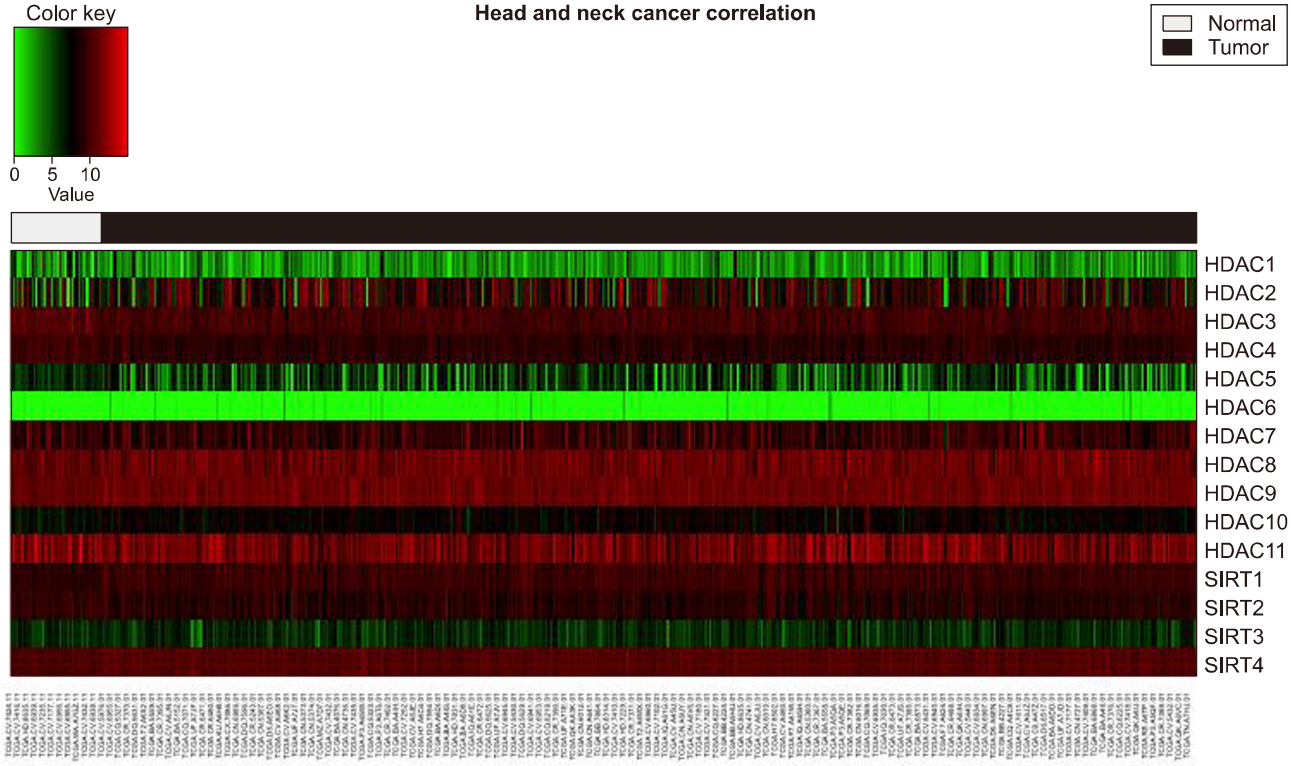

FIG. 1. Heatmap analysis from TCGA data for mRNA expression of HDACs in normal and HNSCC samples.

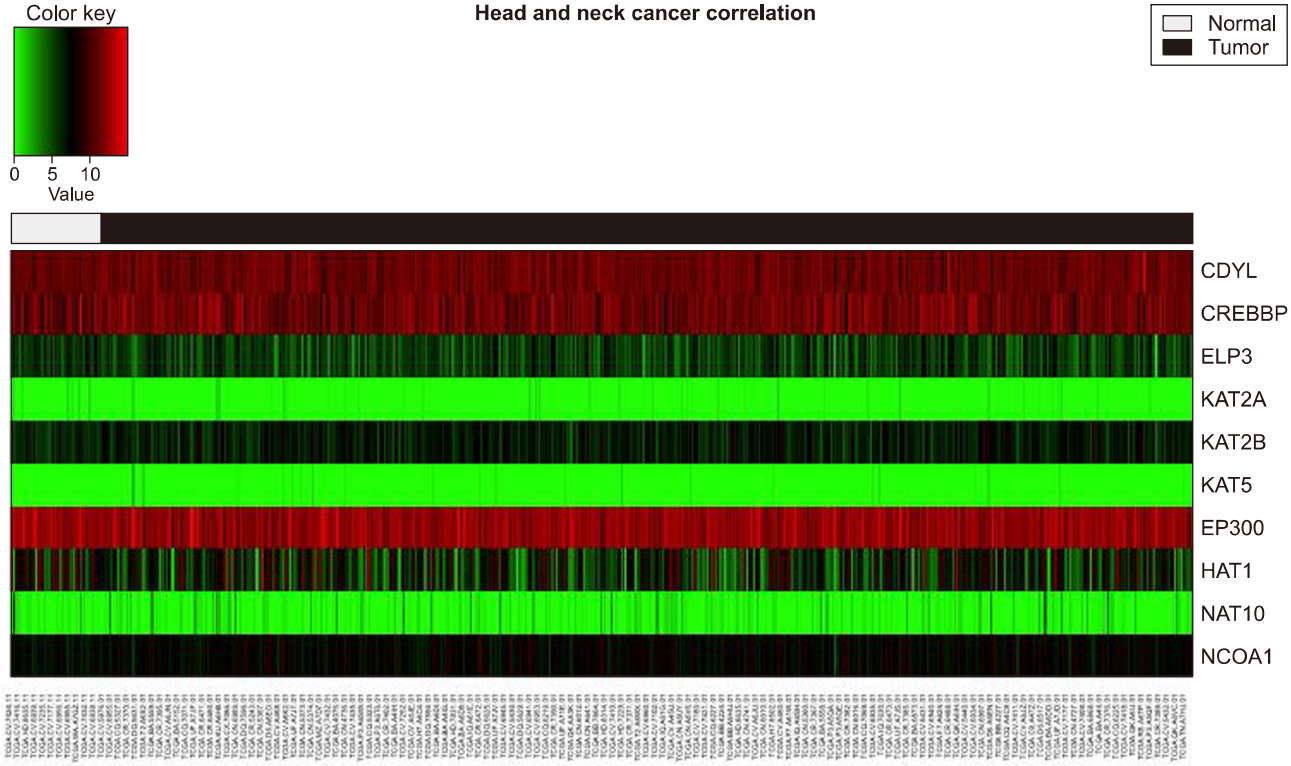

FIG. 2. Heatmap analysis from TCGA data for mRNA expression of HATs in normal and HNSCC samples.

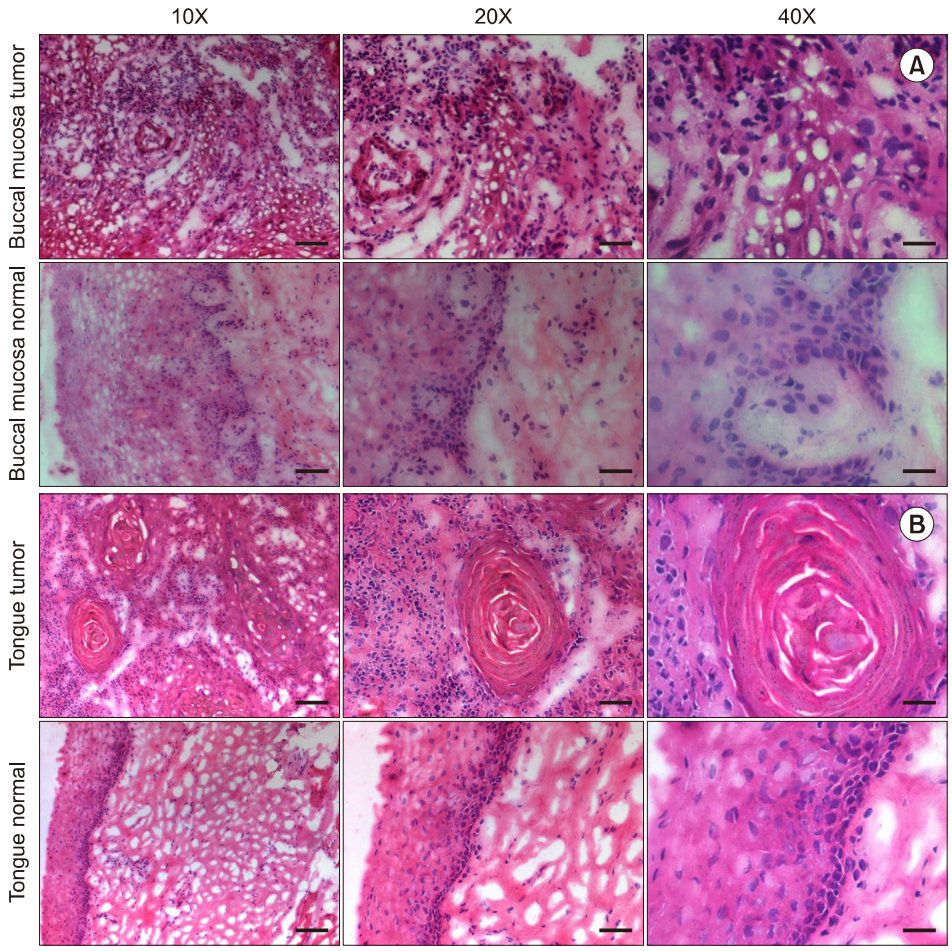

FIG. 3. (A, B) H&E staining of human tissue samples (All scale bars=25 μm).

TABLE 3. RNA yield and quality from tumour samples

Tumour sample	Concentration (ng/μL)	$A_{260/280}$	$A_{260/230}$
Tongue 1	2512	2.04	1.81
Tongue 2	2576	2.03	2.00
Tongue 3	2520	2.05	1.55
Tongue 4	2046	2.06	1.98
Tongue 5	1709	2.04	1.24
Tongue 6	1518	2.06	2.07
Buccal mucosa 1	376.1	2.00	1.24
Buccal mucosa 2	558	2.04	0.69
Buccal mucosa 3	333.7	2.06	0.93
Buccal mucosa 4	360.5	2.08	0.58
Buccal mucosa 5	205.1	2.02	0.50
Buccal mucosa 6	840.4	2.03	1.47

tongue mucosa demonstrated that HDAC2 was upregulated and demonstrated a 4.5 average fold change in these samples. Buccal mucosa samples also demonstrated an overexpression of HDAC2with a 1.5 average fold change in these samples (Fig. 5).

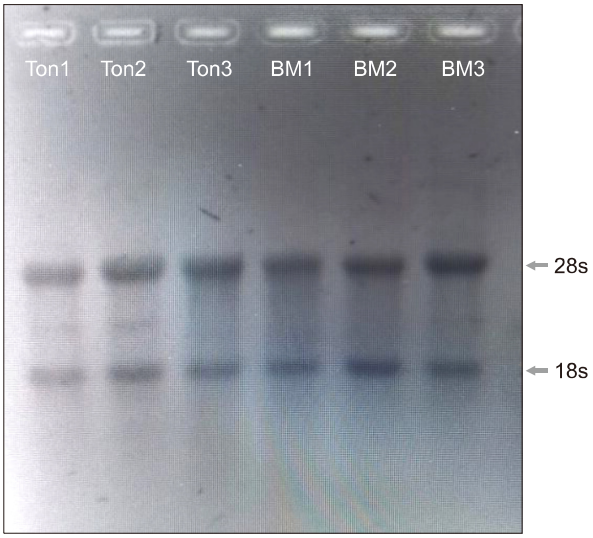

FIG. 4. Resolution of total RNA as seen on agarose gel. Ton: tongue, BM: Buccal mucosa.

DISCUSSION

H and E stain has been traditionally used to stain tissues for microscopic examination. This method provides ex-

cellent distinguishing morphology by contrasting between structures of different composition and allows for combination with further sophisticated methods including immunostaining and *in situ* hybridization.[26] However, H and E staining techniques do not contrast sufficiently between cytoplasm and extracellular structures. Furthermore, cytoplasmic differentiation is insufficient and intracellular glycoproteins, reticular fibers, basement membranes, and cell borders are not stained. Nevertheless, it provides a stable staining pattern in mounted sections and stains the cell nuclei and cytoplasm in strongly dissimilar colors which allows for easy recognition of these structures even under low microscopic magnification.[26]

All samples in this investigation demonstrated diagnostic features of well differentiated, moderately differentiated or poorly differentiated SCC. Well differentiated SCC samples contained squamous cells arranged as islands of different shapes and sizes with keratinous pearls inside. Keratinous pearls contained acidophilic cells with pyknotic nuclei and karolysis. Moderately differentiated OSCC were organized in islands of neoplastic atypical epithelial cells, oval shaped, oblong, round which infiltrated the tumoural stroma. Nuclei of neoplastic cells had different shapes and sizes, most of them hypochromic with large nucleoli. Poorly differentiated SCC appeared as cords, islands or epithelioid-like cells of various shapes and sizes with a complete lack of resemblance to normal epithelium. These histopathological findings when compared with the data obtained from EMR, confirmed the diagnosis of SCC for all the samples.

TABLE 4. Ct value of internal control gene *RPS13*

Tissue samples	Ct value
Tongue 1	20.540
Tongue 1	20.599
Tongue 2	20.956
Tongue 2	20.581
Tongue 3	19.383
Tongue 3	19.592
Buccal mucosa 1	19.449
Buccal mucosa 1	19.003
Buccal mucosa 2	19.078
Buccal mucosa 2	19.552
Buccal mucosa 3	19.597
Buccal mucosa 3	19.522

TCGA database holds a large quantity of high-throughput sequencing and clinicopathological information on different types of cancer. Data from different independent studies can be integrated to obtain a greater number of clinical samples and thus achieve a more robust analysis. However, data from different independent studies may be difficult to aggregate together due to sample heterogeneity, variation in methodology and reporting of results. Nevertheless, efficient integrated bioinformatics methods have been developed for large-scale analysis of cross-platform high throughput data.[27]

In a study of 49 patients with tongue SCC, an over-expression of HDAC1 and HDAC2 in over half of the patients was demonstrated.[19] Interestingly, all the cases showed negative HDAC1 and HDAC2 immunostaining in non-neo-

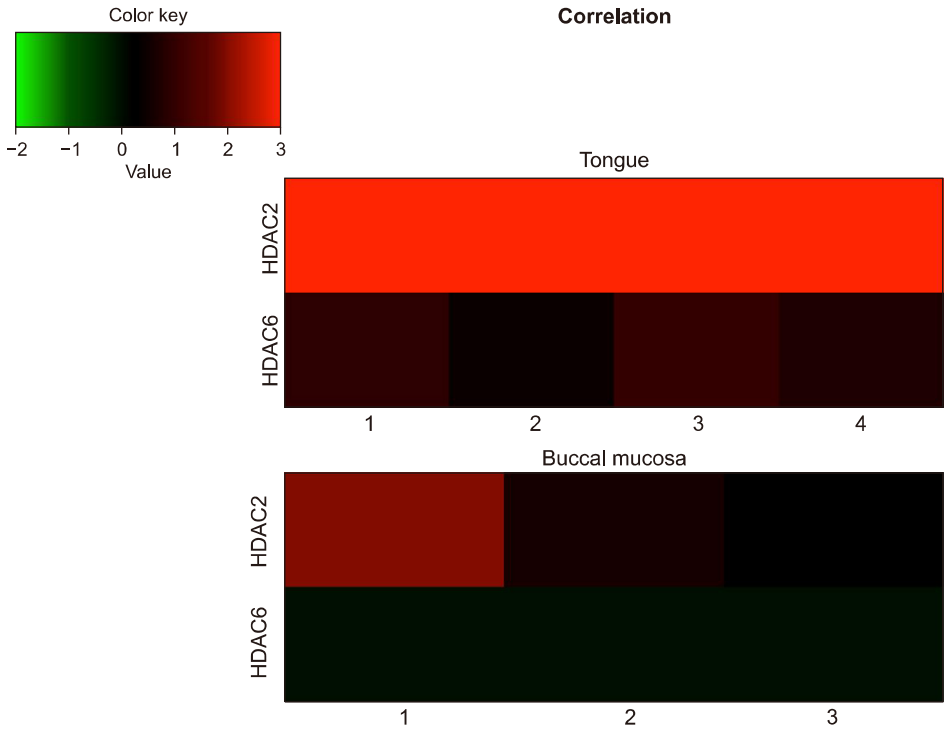

FIG. 5. Heatmap analysis for mRNA expression of HDACs in tongue and buccal mucosa samples.

plastic squamous tongue epithelium. The same study also demonstrated that HDAC1 had a higher clinical value as its overexpression was significantly associated with patient's age, gender, poor histopathological grade of differentiation, presence of lymph node metastases and increased stromal inflammation. In the current study, however, HDAC1 expression levels could not be identified for both buccal mucosa and tongue samples.

HDAC2 overexpression has been found to be significantly associated with the presence and advanced depth of muscular invasion and did not necessarily affect prognosis in a sample of Caucasian population.[19] However, HDAC2 overexpression was identified to be associated with poor prognosis in a group of Taiwanese patients.[20] In the present investigation, HDAC2 levels were also found to be significantly increased and thus confirmed the influence of these epigenetic modifiers in SCC of tongue and buccal mucosa in a cohort of Indian patients. Differential expressions of HDACs within patients highlight the fact that patients should be stratified at a molecular level before providing personalized therapies to achieve optimum treatment success and prognosis. Overall, the overexpression of HDAC2 in all these studies emphasizes the role of this enzyme in epigenetic regulation of tongue and buccal mucosa SCC.

HDAC6 has been found to be overexpressed in advanced stages of HNSCC suggesting that HDAC6 activity may be critical for tumour aggressiveness in oral tumours. HDAC6 can deacetylate α-tubulin increasing cell motility; a fundamental process in the development of tumour metastasis.[21] However, the TCGA analysis demonstrated no change in expression levels of HDAC6 between normal and tumour samples. In this study too, no difference in expressions levels of HDAC6 was noted thus confirming the TCGA data.

Tumours from different anatomic sites have a different genetic constitution and present themselves variably in the clinic. This necessitates a focused approach to each category of cancer even if it is from the same region such us buccal mucosa and tongue that are both a part of the broader HNSCC. Interestingly, tongue SCC has been shown to present the highest rate of failure with poor prognosis compared to other sites of the oral cavity.[28] Furthermore, the ethnicity of the sample population can considerably affect the outcome of the investigation. Subtle epigenetic and genetic variations can significantly alter the role of and influence the action of various human epigenetic modifiers. These changes could account to a considerable extent for the discrepancy in results obtained in different experiments.

Chemotherapy for oral cancers has reduced the number of patients requiring surgical resection and/or radiation therapy.[29,30] Further; advances in combination therapy have contributed to improved cancer treatment modalities. However, drugs used for chemotherapy have a narrow therapeutic index and the 5-year survival rate of patients with OSCC has remained at ~50% for several decades in spite of advancements in chemotherapy, radiotherapy, and surgical approaches.[30] These approaches, although directed towards certain targeted biomolecules, do not discriminate between cancer cells and dividing non-malignant cells and are associated with cytotoxicity. Various clinical side effects of chemotherapy have been reported in the literature particularly with higher doses.[31]

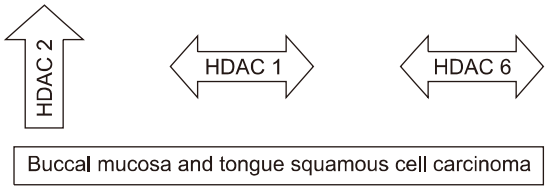

FIG. 6. Graphical summary suggesting increasing of HDAC 2 without change in expression of HDAC 1 & 6 in OSCC.

A number of chemotherapeutic drugs are intercalating agents that wedge between bases along the DNA affecting the structure of DNA, preventing polymerization, and blocking the binding of other proteins to DNA. This results in the prevention of DNA synthesis and inhibition of transcription. Deacetylation of histones by HDACs tightens their interaction with DNA resulting in a closed chromatin organization and inhibition of gene transcription. This mechanism reduces the binding of intercalating molecules to DNA. Histone deacetylase inhibitors (HDACi), the compounds that interfere with the function of HDACs and reverse its action have generated much attention as novel promising epigenetic therapies. These HDACi can open up the chromatin structure and thus facilitate the binding of intercalating drugs to DNA thus promoting the effect of these agents. The complete mechanism by which HDACi effect their action remains incompletely known and depends upon cellular and developmental status.[32] However, the binding of a large number of intercalating molecules to the DNA implies a potent mechanism of action and a reduced dose of chemotherapeutic drugs at a clinical level thus contributing to less cytotoxicity. Besides, normal cells are relatively resistant to treatment with HDACi.[33] Currently, some HDACi for OSCC have been approved for treatment and a number of them are at various stages of clinical trials. Thus, the knowledge of type and expression of HDACs in different cancers is critical to development and selection of HDACi as therapeutic agents.

In spite of the interesting findings, this investigation is not without drawbacks. Only tissue samples that were naïve, free from biological hazards and those that generated significant RNA yield were included in this study. This factor limited the total number of samples significantly. Nevertheless, the validation of observations made *in-silico* with the samples used in this study provides an indication for subsequent targets for therapeutic development.

In conclusion, overexpression of HDAC2 observed *in-silico* was validated in tongue and buccal mucosa SCC samples (Fig. 6). Further studies are need to evaluate gene expression of HDAC2 in a larger number of samples with clinical and demographic data across different populations to allow for the investigation of relevant associations with

clinical outcomes and develop appropriate therapeutic agents targeting these modifiers.

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CONFLICT OF INTEREST STATEMENT

None declared.

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