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Original Article

Status of epigenetic chromatin modification enzymes and esophageal squamous cell carcinoma risk in northeast Indian population

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Abstract: Esophageal cancer incidence is reported in high frequency in northeast India. The etiology is different from other population at India due to wide variations in dietary habits or nutritional factors, tobacco/betel quid chewing and alcohol habits. Since DNA methylation, histone modification and miRNA-mediated epigenetic processes alter the gene expression, the involvement of these processes might be useful to find out epigenetic markers of esophageal cancer risk in northeast Indian population. The present investigation was aimed to carryout differential expression profiling of chromatin modification enzymes in tumor and normal tissue collected from esophageal squamous cell carcinoma (ESCC) patients. Differential mRNA expression profiling and their validation was done by quantitative real time PCR and tissue microarray respectively. Univariate and multiple logistic regression analysis were used to analyze the epidemiological data. mRNA expression data was analyzed by Student t-test. Fisher exact test was used for tissue microarray data analysis. Higher expression of enzymes regulating methylation (DOT1L and PRMT1) and acetylation (KAT7, KAT8, KAT2A and KAT6A) of histone was found associated with ESCC risk. Tissue microarray done in independent cohort of 75 patients revealed higher nuclear protein expression of KAT8 and PRMT1 in tumor similar to mRNA expression. Expression status of PRMT1 and KAT8 was found declined as we move from low grade to high grade tumor. Betel nut chewing, alcohol drinking and dried fish intake were significantly associated with increased risk of esophageal cancer among the study subject. Study suggests the association of PRMT1 and KAT8 with esophageal cancer risk and its involvement in the transition process of low to high grade tumor formation. The study exposes the differential status of chromatin modification enzymes between tumor and normal tissue and points out that relaxed state of chromatin facilitates more transcriptionally active genome in esophageal carcinogenesis.

Keywords: Esophageal cancer, histone modification enzymes, tissue-microarray, KAT8, PRMT1, HDAC

Introduction

An estimated 482,300 new esophageal cancer cases and 406,800 deaths have been reported in 2008 worldwide [1]. The esophageal cancer belt stretches from northern Iran through the central Asian republics to North-Central China and poses to be the geographical region with highest risk where 90% of cases are of squamous cell carcinomas [1]. In India the highest incidence of esophageal cancer is accounted in Northeast population [2]. The etiology is different from other populations in India due to extensive disparities in dietary habits or nutritional factors, tobacco/betel quid chewing and alcohol habits. Tobacco and betel quid (BQ) chewing in this population is the key contributory risk factor of esophageal cancer [2]. BQ chewing habit is mainly reported in India, Sri Lanka, Pakistan, Taiwan, other Southeast Asian countries and South Africa [3]. BQ usually comprises a piece of areca nut, inflorescence *Piper betle*, and lime with or without *Piper betle* leaves [3, 4]. Alkaloids present in the areca nut are found cytotoxic and genotoxic to various cells in vitro [3]. Arecoline is a major component

of areca nut and is found to be mutagenic to mammalian cells and causes chromosomal aberration in Chinese hamster ovary cells or mouse bone marrow cells [5-7].

A recent report from our group stats that genetic variants of tobacco carcinogens metabolizing microsomal epoxide hydrolase 1 (EPHX1) gene are associated with a high risk of esophageal cancer in northeast Indian population [8]. In another study the immunohistochemical expression of cytokeratins in normal esophageal epithelium and esophageal squamous cell carcinoma from both the general population from Delhi and the high-risk population of Assam, Northeast India is reported [9]. The results suggest that CK5 and CK8 expression might be useful markers for separating high-risk and low risk population groups [9]. Cytokeratins are the major constituents of the esophageal epithelium and may show gain or loss as the cancer progresses from normal epithelium to invasive phenotype. Additionally, we have earlier described several differentially expressed genes in familial and non-familial esophageal cancer patients that have also been reported from a high-incidence region of esophageal cancer in China [4, 10]. Genome-wide analysis of chromosomal alterations in ESCC patients exposed to tobacco and BQ has also been previously done using Affymetrix 10K SNP arrays [11].

Post translational modifications of histone proteins alter the gene expression and are catalyzed by chromatin modification enzymes, consequently the expression status of these enzymes might be beneficial to find out epigenetic markers of esophageal cancer risk in high risk population of northeast India. A complex interplay of both tumor suppressor genes promoter methylation and their histone modifications status is also reported that maintains the active status of these genes in malignancies [12]. Furthermore promoter DNA methylation inhibits gene expression either by hindering the association of some transcription factors with their cognate DNA recognition sequences or by binding of methyl binding proteins (MBD1, MBD2, MBD3 and MeCP2) which in turn recruit histone modification enzymes thus transcriptionally silencing the genomic region [13, 14]. Moreover MeCP2 allegedly represses transcription of methylated DNA through the recruitment of a histone deacetylase (HDAC)-containing complex [15, 16], thus paying stress to the role of histone modification enzymes in DNA methylation mediated epigenetic silencing of genes. Consequently, the present study is intended to carry out differential mRNA expression analysis of genes that code enzymes involved in DNA methylation and histone tail modifications by real time PCR arrays along with their validation by tissue microarray (TMA) based immunohistochemistry in tumor and normal tissue samples of ESCC.

Materials and methods

Sample collection

Tumor and normal tissue were collected from newly diagnosed esophageal cancer patients at Dr. Bhubaneshwar Borooah Cancer Institute (BBCI), Guwahati, Assam, India. The study had been approved by institutional ethics committee at BBCI and a well-informed written consent was taken prior to collection of the endoscopic biopsies from patients. A part of each collected tissue was preserved in formalin for histopathologic examination at BBCI. The remaining tissue was immediately collected in RNA later solution (Ambion, Austin, USA) and stored at -70°C until processed. Total 110 patients of esophageal cancer were recruited. Majority of the cases both male and female were in the age group of 51-60. Histopathologic examination revealed that majority of the cases were of squamous cell carcinoma (90.0%; 99/110) followed by Adenocarcinoma (10.0%; 11/110). All ninety nine histopathologically confirmed ESCC cases were considered for the present study. A detailed questionnaire regarding information about patient's dietary habits, lifestyle factor as well as family history of esophageal cancer was filled at the time of sample collection. The positive family history of esophageal cancer was taken as exclusion criteria. Northeast patients of the same ethnic group residing in northeast states of India since last 25 years were considered for the study and it was strictly used as an inclusion criteria. Seventy five sex and ethnicity matched control individuals of similar age group (± 5 years) were also included to investigate the comparative epidemiological parameter between control and patients. Controls also did not have any history of cancer and written consent was also taken from them. Comparative tobacco chewing and smoking status, betel nut chewing status and alcohol consumption status of patient and controls were evaluated (**Table 1**).

Table 1. Risk estimation according to dietary (Smoked food, Pickled food, Dried fish, Fresh fish and Green-leafy vegetables) and habitual (Betel nut and tobacco chewing, tobacco smoking and alcohol habits) factors in north east population

Category	Case *n* (%)	Control *n* (%)	Crude OR (95% CI)	*p*-value	Multivariate OR (95% CI)	*p*-value
Smoked food						
Non-consumers	32 (42.7)	45 (60.0)	Reference	0.034	Reference	0.092
Consumers	43 (57.3)	30 (40.0)	2.02 (1.05-3.86)		1.05 (0.44-2.50)	
Pickled food						
Non-consumers	32 (42.7)	34 (45.3)	Reference	0.742	Reference	0.896
Consumers	43 (57.3)	41 (54.7)	1.11 (0.59-2.12)		1.05 (0.53-2.08)	
Dried fish						
Non-consumers	31 (41.3)	50 (66.7)	Reference	0.002	Reference	0.025*
Consumers	44 (58.7)	25 (33.3)	2.84 (1.46-5.52)		2.77 (1.14-6.74)	
Fresh fish						
Non-consumers	7 (9.3)	9 (12.0)	Reference	0.597	Reference	0.697
Consumers	68 (90.7)	66 (88.0)	1.33 (0.47-3.76)		0.78 (0.22-2.73)	
Green-leafy Vegetables						
Non-consumers	5 (6.7)	8 (10.7)	Reference	0.384	Reference	0.683
Consumers	70 (93.3)	67 (89.3)	1.67 (0.52-5.37)		1.35 (0.32-5.60)	
Betel-nut Chewing status						
Non chewer	7 (9.3)	23 (30.7)	Reference	0.001	Reference	0.046*
Ever chewer	68 (90.7)	52 (69.3)	4.30 (1.71-10.78)		2.79 (1.02-7.62)	
Tobacco Chewing status						
Non chewer	25 (33.3)	42 (56.0)	Reference	0.005	Reference	0.162
Ever chewer	50 (66.7)	33 (44.0)	2.55 (1.31-4.94)		1.76 (0.80-3.91)	
Tobacco Smoking status						
Non smoker	26 (34.7)	34 (45.3)	Reference	0.182	Reference	0.080
Ever smoker	49 (65.3)	41 (54.7)	1.56 (0.81-3.02)		1.93 (0.92-4.03)	
Alcohol Drinking status						
Non drinker	34 (45.3)	53 (70.7)	Reference	0.002	Reference	0.33*
Ever drinker	41 (54.7)	22 (29.3)	2.91 (1.48-5.70)		2.21 (1.07-4.57)	

*Significant *i.e* indicates $p \leq 0.05$.

RNA isolation

Total RNA was extracted from tissue samples using RNeasy Mini Kit (QIAGEN, Hilden, Germany) following manufacturer's protocol. RNA was re-suspended in nuclease free water at a concentration of 100-150 ng/µl with the A260/A280 ratio between 2.0-2.2. Total RNA was used immediately for cDNA preparation by using cDNA synthesis kit (QIAGEN, Hilden, Germany) following manufacturer's protocol. The cDNA was used for differential expression profiling by RT^2 Profiler™ PCR array of human epigenetic chromatin modification enzymes through Real Time Thermal Cycler ABI 7000 (Applied Biosystem, USA).

Quantitative PCR

The differential mRNA expression profiling of Epigenetic Chromatin Modification Enzymes in pooled normal and tumor tissue was done by RT^2 Profiler™ PCR Array Human Epigenetic Chromatin Modification Enzymes from QIAGEN. PCR-array profiled the expression of 84 key genes encoding enzymes known or predicted to modify genomic DNA and histones to regulate chromatin accessibility and therefore gene expression. The de novo and maintenance DNA methyltransferases, and the enzymes responsible for demethylation of CpG dinucleotides were represented by the array. Enzymes catalyzing histone acetylation, methylation, phos-

phorylation, and ubiquitination were also included on the array as well as the deacetylases and demethylases. The array also analyzed genes encoding the SET domain proteins, which all contain a homologous domain that demonstrates histone methyltransferase activity in some family members. For the normalization of the expression data the 96-well PCR array contained beta actin (ACTB), Beta-2-microglobulin (B2M), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Hypoxanthine phosphoribosyltransferase 1(HPRT1) and Ribosomal protein, large, P0 (RPLP0). Additionally PCR array also contained Reverse Transcription Control and Positive PCR Control probes to check the efficiency of assay. To test the genomic DNA contamination in the sample the PCR array contained a Human Genomic DNA Contamination probe.

Due to the less amount of tissue in each endoscopic biopsy 4 tissue samples were pooled from patients having matched sex, age, type and grade of cancer for each experiment. Total 6 experiments with tumor tissue samples and 6 with normal tissue samples were done. Pooling was done among samples collected from patients with moderately differentiated squamous carcinoma with similar histopathological grade G2. Isolated RNA was immediately converted into cDNA by QUIGEN cDNA synthesis kit following manufacturer's protocol. Differential mRNA expression profiling was done using PCR array on Applied Biosystem real time thermal cycler (ABI 7000). The threshold cycle (Ct) value was recorded for each gene and was used for calculation of fold change in gene expression by $\Delta\Delta C_T$ data analysis method. Fold-Change [2^(- Delta Delta Ct)] is the ratio of normalized gene expression [2^(- Delta Ct)] in the tumor and normalized gene expression [2^(- Delta Ct)] in the normal tissue sample. Fold-regulation was used to represent fold-change results in a biologically meaningful way. Fold-change values greater than one indicate a positive or an up-regulation, and in this case the fold-regulation is equal to the fold-change. However, less than one fold-change values indicate a negative or down-regulation, and in this case the fold-regulation is the negative inverse of the fold-change.

Tissue microarray (TMA) based immunohistochemistry

For TMA a separate cohort of samples comprising 75 ESCC and 20 non-neoplastic control tissue samples were used. The cohort comprised of patients having different grade of ESCC i.e. well differentiated (Grade 1), moderately differentiated (Grade 2) and poorly differentiated (Grade 3). A TMA was constructed from the formalin-fixed, paraffin-embedded blocks of these tissue samples to validate the results of differential mRNA expression analysis of histone modification enzymes (KAT8, KAT2A and PRMT1) at protein level. Sampling sites were marked on the donor blocks and the tissue cylinders were precisely arrayed into two recipient blocks, each with a core size of 1.5 mm using semi-automatic tissue microarrayer (Alphelys, SAS, France). TMA block had 20 non-neoplastic esophageal epithelium taken from distant sites (control) and 75 samples from ESCC. Poly-L-lysine coated slides were incubated overnight at room temperature and deparaffinized in 2 changes of xylene and rehydrated in graded alcohol. Antigen retrieval was done in Tris-EDTA (pH 9.0) buffer at 90°C for 20 minutes. The sections were incubated in wash buffer TBS (pH 7.4, 50 mM Tris (Sigma-Aldrich) and 150 mM NaCl) for 5 minutes. Endogenous peroxidase blocking was done in 3% H_2O_2 for 10 minutes. After washing the sections were incubated with protein blocking (Dako) for 10 minutes followed by incubation with primary antibodies (Abcam) in humidity chamber overnight at 4°C. For this rabbit polyclonal KAT8 and PRMT1 primary antibodies were used in working dilution of 1:100. Similarly mouse monoclonal KAT2A primary antibody was also used in working dilution of 1:100. After incubation with primary antibody sections were washed in TBS with three changes for 5 minutes each. Sections were incubated with HRP tagged secondary antibody (polymer, Dako) for 30 minutes at room temperature in humidity chamber. After washing reactions were developed with diaminobenzidine and rinsed with PBS followed by counter staining with hematoxylin. TMA slides were then scanned by Digital Scanning Microscope (MetaSystems) and images were taken at 10X magnification. Staining was scored semi-quantitatively as < 10% or no staining = 0, 10-40% = 1 and 41-100% = 2 and termed as non-reactive, weak immunoexpression and strong immunoexpression respectively. Frequency of different intensity scores were calculated in control and ESCC samples. As well as frequency of different intensity scores were also calculated in different grades of the ESCC i.e. well differentiated (Grade 1), moder-

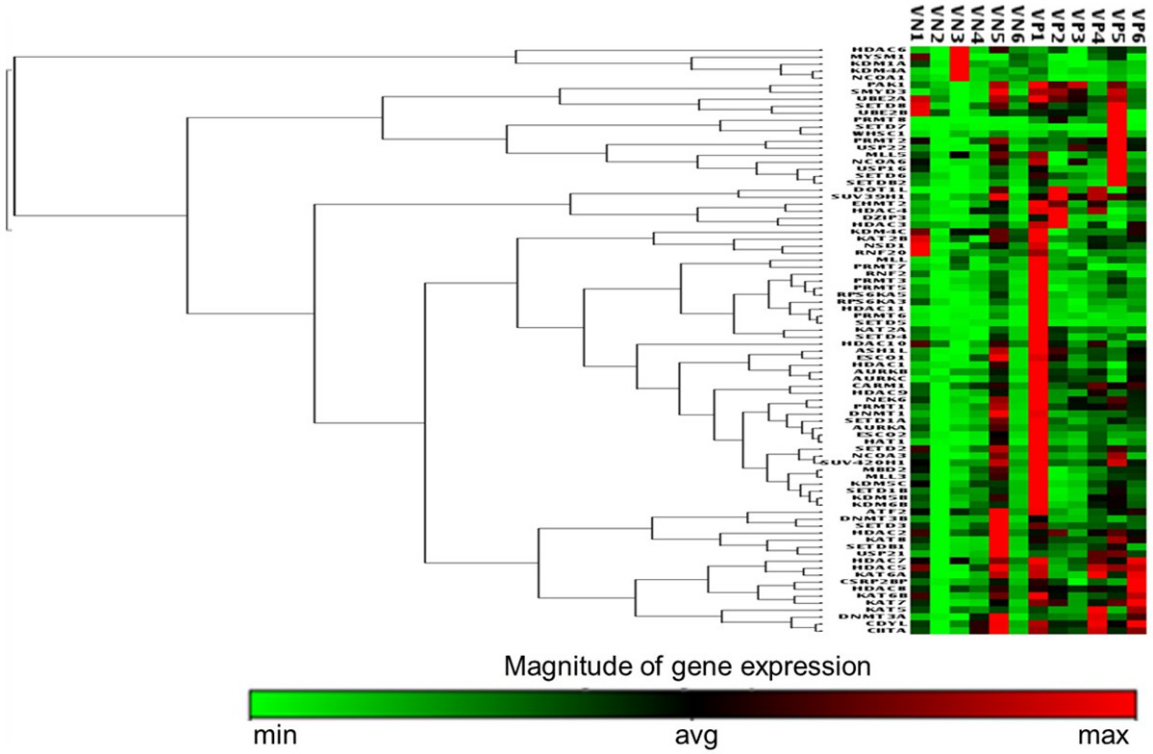

Figure 1. Differential mRNA expression of chromatin modification enzymes. Non-supervised hierarchical clustering of the entire dataset displaying a heat map with dendrograms indicating co-regulated genes across pooled samples of tumor and adjacent normal tissue. VN1 to VN6 represent pooled normal tissue samples and VP1 to VP6 represent pooled tumor tissue samples.

ately differentiated (Grade 2) and poorly differentiated (Grade 3).

Statistical analysis

The fold change in mRNA expression between tumor and normal tissue for each gene was calculated by $\Delta\Delta C_T$ data analysis method through Microsoft excel template based calculations provided by QIAGEN. The graphical representation of expression data was done by web based methods freely available at QIAGEN site. Due to the inverse proportional relationship between the threshold cycle (Ct) and the original gene expression level, and the doubling of the amount of product with every cycle, the original expression level (L) for each gene of interest was expressed as $L = 2^{-Ct}$. To normalize the expression level of a gene of interest (GOI) to a housekeeping gene (HKG), the expression levels of the two genes were divided as $2^{-Ct\,(GOI)}/2^{-Ct\,(HKG)} = 2^{-Ct\,(GOI)\,-\,Ct\,(HKG)} = 2^{-\Delta Ct}$. To determine fold change in gene expression, the normalized expression of the GOI in the tumor sample was divided by the normalized expression of the same GOI in the normal sample as: $2^{-\Delta Ct\,(Tumor)}/2^{-\Delta Ct\,(Normal)} = 2^{-\Delta\Delta Ct}$. Where $\Delta\Delta Ct$ is equal to ΔCt (Tumor) – ΔCt (Normal).

Significant change in mRNA expression between normal and tumor groups were analyzed by Student t-test. TMA data was analyzed by Fisher exact test as the cell frequencies were found less than 5 in control samples as none of the control sample showed intensity score 1 and 2 for KAT8 and PRMT1. Similarly for KAT2A intensity score 0 was found only in 1 control samples. P value ≤ 0.05 was considered significant.

Results

Epidemiological factors

Frequency of male cases (77.3%, 58/75) were reportedly higher than the female cases (22.7%, 17/75). Majority of the cases both male and female were in the age group of 51-60. According to histological grades majority of the cases were moderately differentiated Grade 2 (66.7%; 50/75) followed by well differentiated Grade 1 (18.7%; 14/75) and poorly differenti-

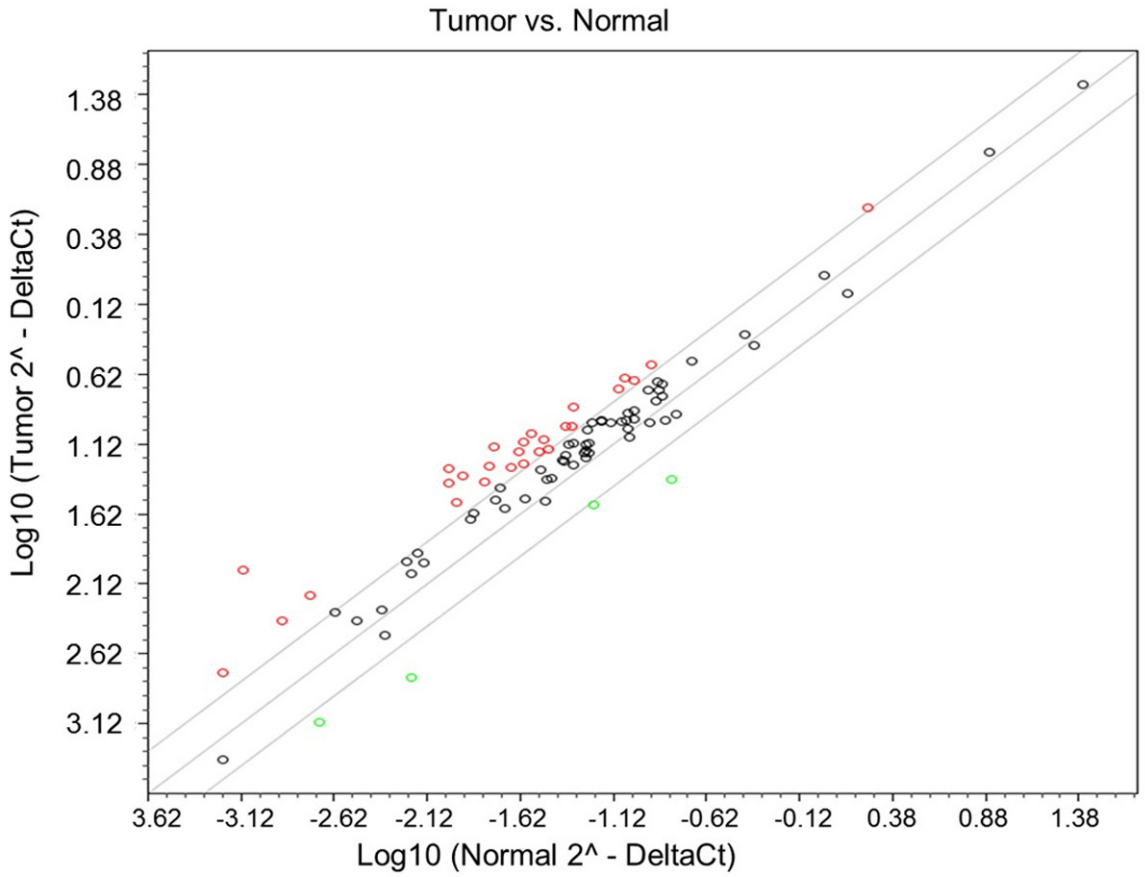

Figure 2. The scatter plot showing the normalized expression of every gene on the array between two groups (normal and tumor tissue). The central line indicates unchanged gene expression. The boundaries or the fold regulation cut-off is 2. Red and green dots represent genes up regulated and down regulated in tumor tissue compare to adjacent normal tissue respectively.

ated Grade 3 (14.6%; 11/75). Betel nut chewing was significantly associated with increased risk of esophageal cancer among the study subject (OR = 2.79, CI = 1.02-7.622; p = 0.046) (**Table 1**). Tobacco chewing and tobacco smoking was found to confer a risk but it was not found to be significant (OR = 1.76, CI = 0.80-3.91; p = 0.162 for tobacco chewing and OR = 1.93, CI = 0.92-4.03; p = 0.080 for tobacco smoking). Alcohol drinking was significantly associated with esophageal cancer risk (OR = 2.21, CI = 1.07-4.57; p = 0.033). Significant risk association was observed for the intake of dried fish among the study subject (OR = 2.77, CI = 1.14-6.74; p = 0.025) (**Table 1**). No association was observed for intake of smoked food, pickled food, and fresh fish.

mRNA expression of chromatin modification enzymes

Among the genes analyzed only 30 showed more than two fold up or down regulation (**Figures 1-3**). DOT1L gene (DOT1-like, histone H3 methyltransferase (*S. cerevisiae*) showed 12.26 fold up-regulation of mRNA expression in tumor tissues compared to normal tissue samples with marginal significance (p-value 0.05). Although differentially upregulated in tumor tissue normalized DOT1L expression level was low in both the tumor and normal tissues in comparison to other histone modification enzymes included in the study. Nuclear receptor co-activator 1 (NCOA1) gene showed lowest - 3.8544 fold down regulation in mRNA expression in tumor tissue but the change was not found statistically significant. DOT1L, KAT7, SMYD3, KAT-2A, NEK6, ASH1L, RPS6KA5, EHMT2, ESCO1, KAT8, PRMT1, SETD1A and AURKC genes were found significantly up regulated in tumor tissues (**Figure 4**). Average normalized expression of PRMT6, HAT1, HDAC11 and DZIP3 were also found higher in tumor tissues compared to normal but the results were not found statistically significant. The higher expression of another

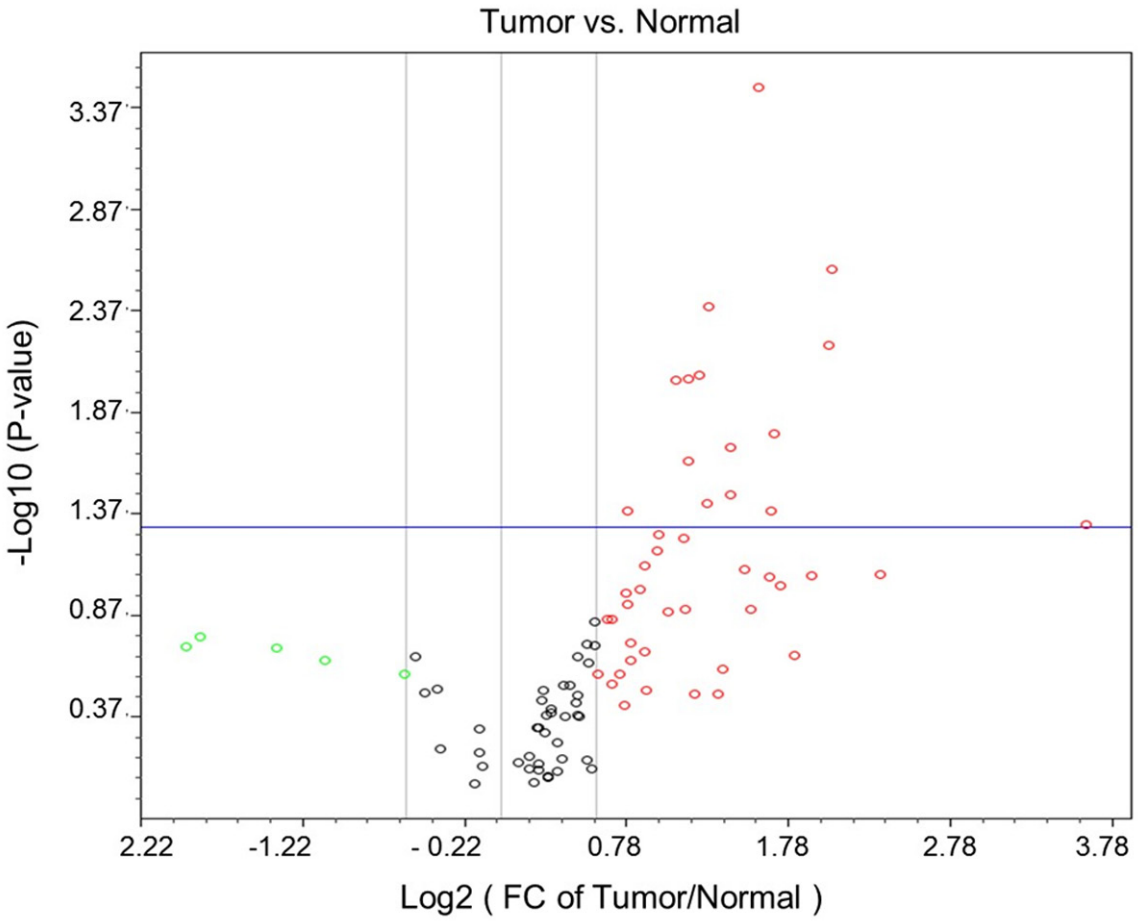

Figure 3. Significant differential mRNA expression of chromatin modification enzymes. The volcano plot displaying statistically significant (p-value ≤ 0.05) fold regulation in mRNA expression of chromatin modification enzymes. The plot displays statistical significance versus fold regulation on the y- and x-axes respectively. The boundaries or the fold regulation cutoff is 2 and a p-value cutoff value is 0.05. Red and green dots represent genes up regulated and down regulated in tumor tissue compare to adjacent normal tissue respectively. Red dots above the blue line showing statistically significant (p-value ≤ 0.05) up-regulated gene expression.

important histone modification enzyme HDAC9 in tumor tissue was observed but it showed only marginal statistical significance (p-value 0.053). KDM4A, MYSM1, KDM1A and NCOA1 showed decreased level of mRNA expression in tumor tissue (**Table 2**). None of the down regulated genes was found statistically significant although KDM1A and NCOA1 expression were found more than 3 fold down regulated in tumor tissue. Statistically significant differential mRNA expression of genes was diagrammatically represented in **Figure 5**.

Protein expression of KAT8, PRMT1 and KAT2A in control and ESCC

Differential protein expression of KAT8, PRMT1 and KAT2A was also checked in ESCC tissues by TMA based immunohistochemistry analysis. Total 20 controls and 75 squamous carcinoma tissue samples were used. Some of the tissue sections were not considered for immunohistochemical analysis as some cores were not found suitable for interpretation. Nuclear non-reactive, weak immunoexpression and strong immunoexpression score for KAT8 were found in 32, 34 and 4 tumor samples respectively. Similarly for PRMT1 non-reactive, weak immunoexpression and strong immunoexpression score were found in 31, 13 and 26 tumors respectively. However, all 15 control tissues were found non-reactive for KAT8 and PRMT1 nuclear expression (**Figure 6**). KAT2A (GCN5) cytoplasmic non-reactive, weak and strong immunoexpression score were found in 8, 37 and 25 tumors respectively (**Figure 7**). In con-

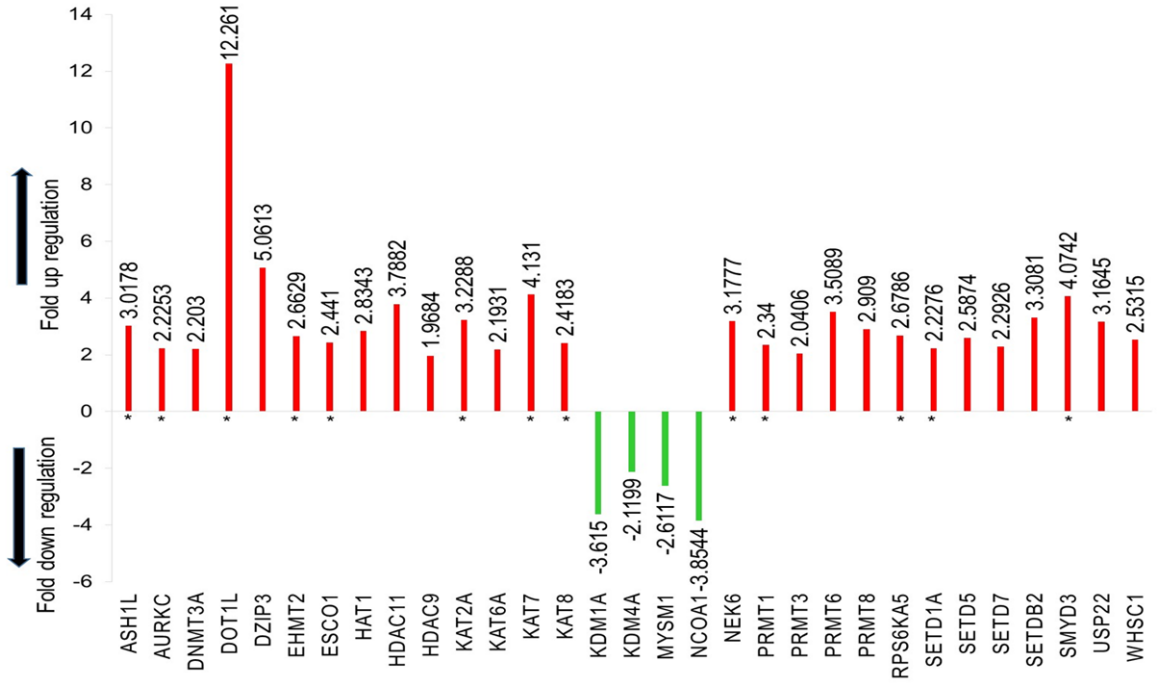

Figure 4. Fold change in mRNA expression. Histone modification enzymes showing more than 2 fold change in expression levels in tumor tissue compared to normal tissue. Red color bar showing up-regulation and green color showing down regulation in expression level in tumor tissue. *representing the enzymes having fold change with statistical significance of *p*-value < 0.05.

Table 2. List of genes showing fold regulation in mRNA expression. Genes coding chromatin modification enzymes that have showed more than two fold up-regulation or down-regulation in mRNA expression in tumor tissues compared to adjacent normal tissues are listed. Most of the differentially expressed genes were up-regulated however only four genes were found down-regulated in tumor tissue compared to adjacent normal tissue

Symbol	Description	Fold Regulation	*P*-value
ASH1L	Ash1 (absent, small, or homeotic)-like (Drosophila)	3.0178	0.00034
AURKC	Aurora kinase C	2.2253	0.023647
DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha	2.203	0.125805
DOT1L	DOT1-like, histone H3 methyltransferase (S. cerevisiae)	12.261	0.048491
DZIP3	DAZ interacting protein 3, zinc finger	5.0613	0.08503
EHMT2	Euchromatic histone-lysine N-methyltransferase 2	2.6629	0.020146
ESCO1	Establishment of cohesion 1 homolog 1 (S. cerevisiae)	2.441	0.00407
HAT1	Histone acetyltransferase 1	2.8343	0.080194
HDAC11	Histone deacetylase 11	3.7882	0.08619
HDAC9	Histone deacetylase 9	1.9684	0.053835
KAT2A	K(lysine) acetyltransferase 2A	3.2288	0.017306
KAT6A	K(lysine) acetyltransferase 6A	2.1931	0.056167
KAT7	K(lysine) acetyltransferase 7	4.131	0.002656
KAT8	K(lysine) acetyltransferase 8	2.4183	0.037913
KDM1A	Lysine (K)-specific demethylase 1A	-3.615	0.172096
KDM4A	Lysine (K)-specific demethylase 4A	-2.1199	0.226517
MYSM1	Myb-like, SWIRM and MPN domains 1	-2.6117	0.194962
NCOA1	Nuclear receptor coactivator 1	-3.8544	0.194186
NEK6	NIMA (never in mitosis gene a)-related kinase 6	3.1777	0.041111

PRMT1	Protein arginine methyltransferase 1	2.34	0.008843
PRMT3	Protein arginine methyltransferase 3	2.0406	0.13052
PRMT6	Protein arginine methyltransferase 6	3.5089	0.214214
PRMT8	Protein arginine methyltransferase 8	2.909	0.126327
RPS6KA5	Ribosomal protein S6 kinase, 90kDa, polypeptide 5	2.6786	0.034466
SETD1A	SET domain containing 1A	2.2276	0.009203
SETD5	SET domain containing 5	2.5874	0.248734
SETD7	SET domain containing (lysine methyltransferase) 7	2.2926	0.328293
SETDB2	SET domain, bifurcated 2	3.3081	0.096837
SMYD3	SET and MYND domain containing 3	4.0742	0.006322
USP22	Ubiquitin specific peptidase 22	3.1645	0.08773
WHSC1	Wolf-Hirschhorn syndrome candidate 1	2.5315	0.330608

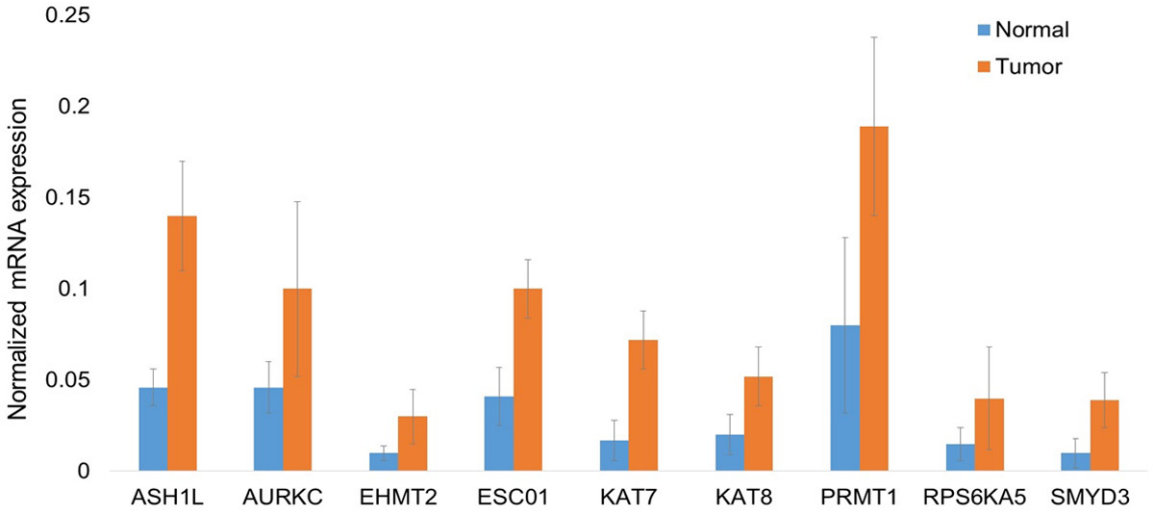

Figure 5. Differential mRNA expression. Normalized expression levels of selected genes in tumor tissue and normal tissue that were found differentially expressed with statistical significance of *p*-value < 0.05.

trols KAT2A non-reactive, weak and strong immunoexpression score were found in 1, 9 and 5 samples (**Figure 7**). The significant higher frequencies of strong and weak immunoexpression scores for PRMT1 were found in tumors compared to controls (**Figure 8**). Similarly significant higher frequency of weak immunoexpression score for KAT8 was found in tumors (**Figure 8**). Frequency of intensity scores for KAT2A were not found significantly altered between tumor and controls (**Figure 8**). Collectively histone modification enzymes; KAT8 and PRMT1 showed significant higher protein expression in tumors as assumed by their higher mRNA expression found in quantitative RT-PCR experiments. Although at mRNA level KAT2A was found up-regulated in tumor, protein expression was not found significantly altered between tumor and controls.

Protein expression of KAT8, PRMT1 and KAT2A in different grades of ESCC

Frequency of non-reactive, weak immunoexpression and strong immunoexpression scores were also calculated in different grades of the ESCC i.e. well differentiated (Grade 1), moderately differentiated (Grade 2) and poorly differentiated (Grade 3) for KAT8, PRMT1 and KAT2A. The frequency of weak immunoexpression score of KAT8 was found significantly lower in Grade 3 (0; 0/10) compared to Grade 1 (0.43; 3/7) and Grade 2 (0.36; 10/28) ESCC. Similarly frequency of weak immunoexpression score of PRMT1 was found lower in Grade 3 (0.11; 1/9) compared to Grade 1 (0.37; 3/8) and Grade 2 (0.28; 7/25) but was not statistically significant. However, frequency of weak and strong immunoexpression scores of KAT2A was not found

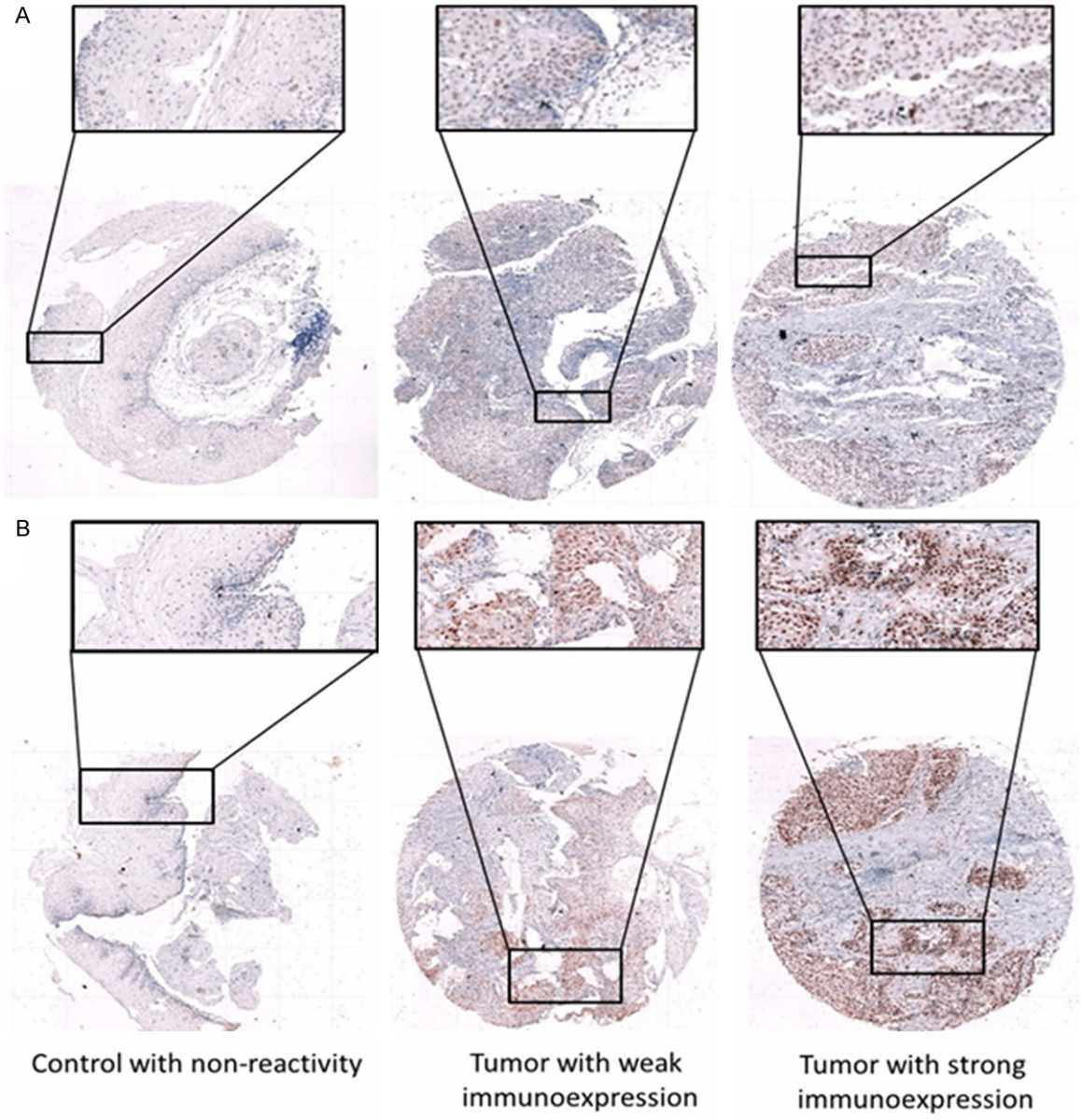

Figure 6. Tissue microarray based immunohistochemistry for KAT8 and PRMT1. Tissue microarray images of (A) KAT8 and (B) PRMT1 showing no nuclear reactivity in control epithelium whereas weak and strong nuclear expression respectively in ESCC. Zoom image of selected region from the core (inset).

significantly altered among different grades of ESCC (**Figure 9**).

Discussion

Chromatin remodeling of a cell depends on the histone proteins posttranslational modifications. These changes are catalyzed by number of enzymes; therefore the status of these enzymes at the level of mRNA was examined between normal and tumor tissue of squamous esophageal cancer patients. Our data clearly show higher expression of histone lysine methyltransferases (DOT1L, SMYD3) and lysine acetyltransferases (KAT2A, KAT6A, KAT7 and KAT8) along with higher arginine methyltransferases (PRMT1, PRMT3, PRMT6 and PRMT8) expression in tumor tissue. Whereas the lysine demethylases (KDM1A and KDM4A) expression was found down regulated in tumor tissue that shows that histone methylation at lysine residues possibly be associated with increased

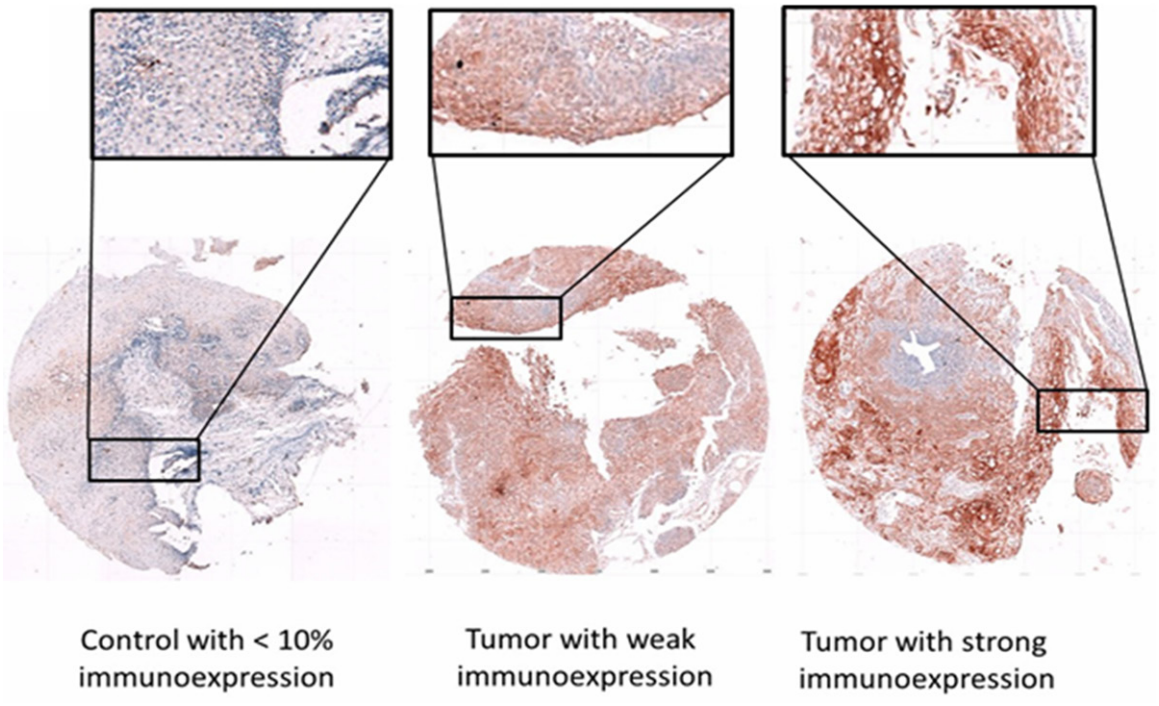

Figure 7. Tissue microarray based immunohistochemistry for KAT2A. Tissue microarray images of KAT2A in control epithelium and ESCC showing no cytoplasmic immunoreaction, weak immunoreaction and strong immunoreaction, respectively. Zoom of selected region from the core (inset).

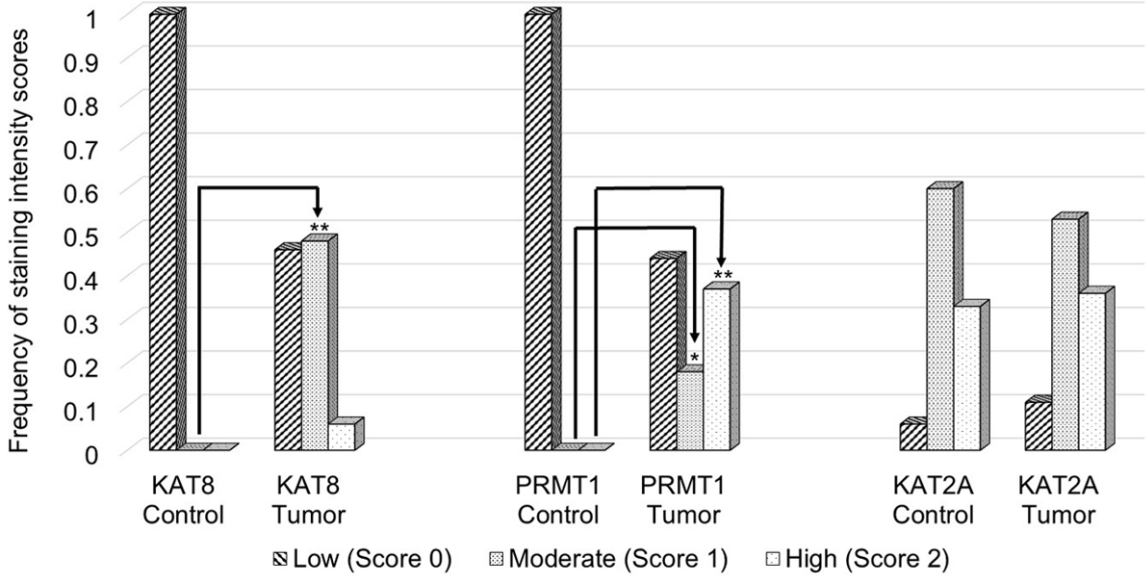

Figure 8. Differential immunoreactivity in ESCC. Bar diagrammatic representation of frequency of TMA based immunohistochemistry staining intensity scores 0, 1 and 2 for KAT8, PRMT1 and KAT2A in tumor and control samples. Low, moderate and high intensities were scored as 0, 1 and 2 respectively. Significant change in frequency of intensity score in tumor in comparison to respective control score showed by *representing *p*-value < 0.05 and **representing *p*-value < 0.01.

esophageal cancer risk in northeast Indian population. Histone acetylation is often reported to be associated with a more "open" chromatin conformation. Chromatin immunoprecip-

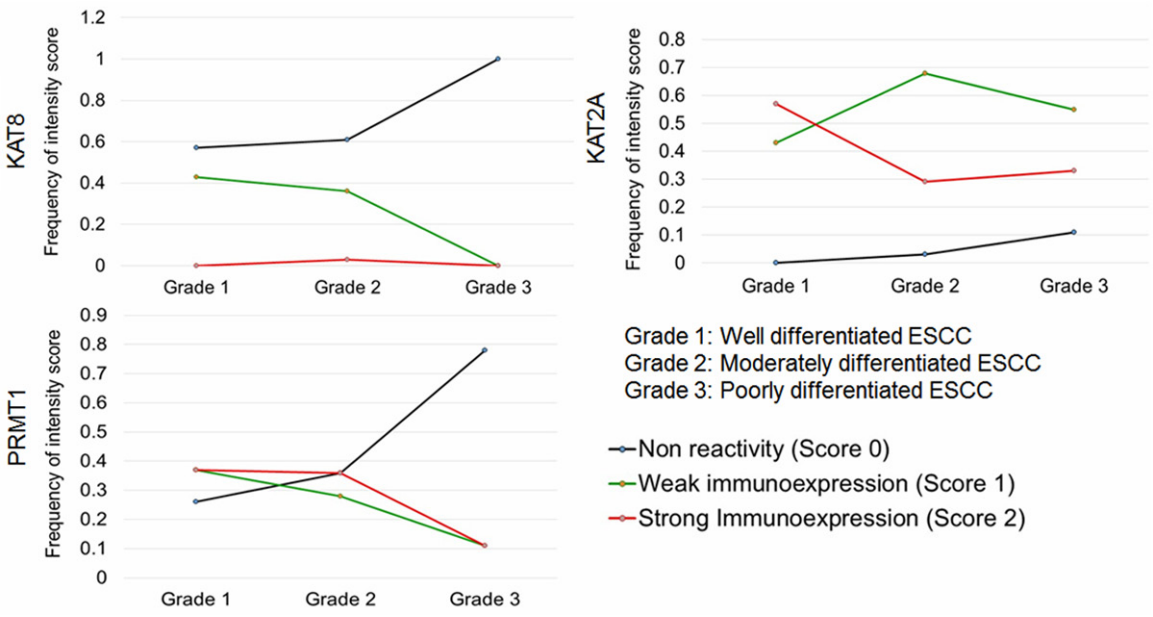

Figure 9. Quantification of immunoreactivity in different grades of ESCC. Line diagram showing frequency of staining intensity scores for KAT8, PRMT1 and KAT2A expression in different grades of ESCC. Black, Green and Red Lines representing non reactivity (score 0), weak immunoexpression (Score 1) and strong immunoexpression (Score 2) respectively. Grade 1, Grade 2 and Grade 3 are well differentiated, moderately differentiated and poorly differentiated ESCC.

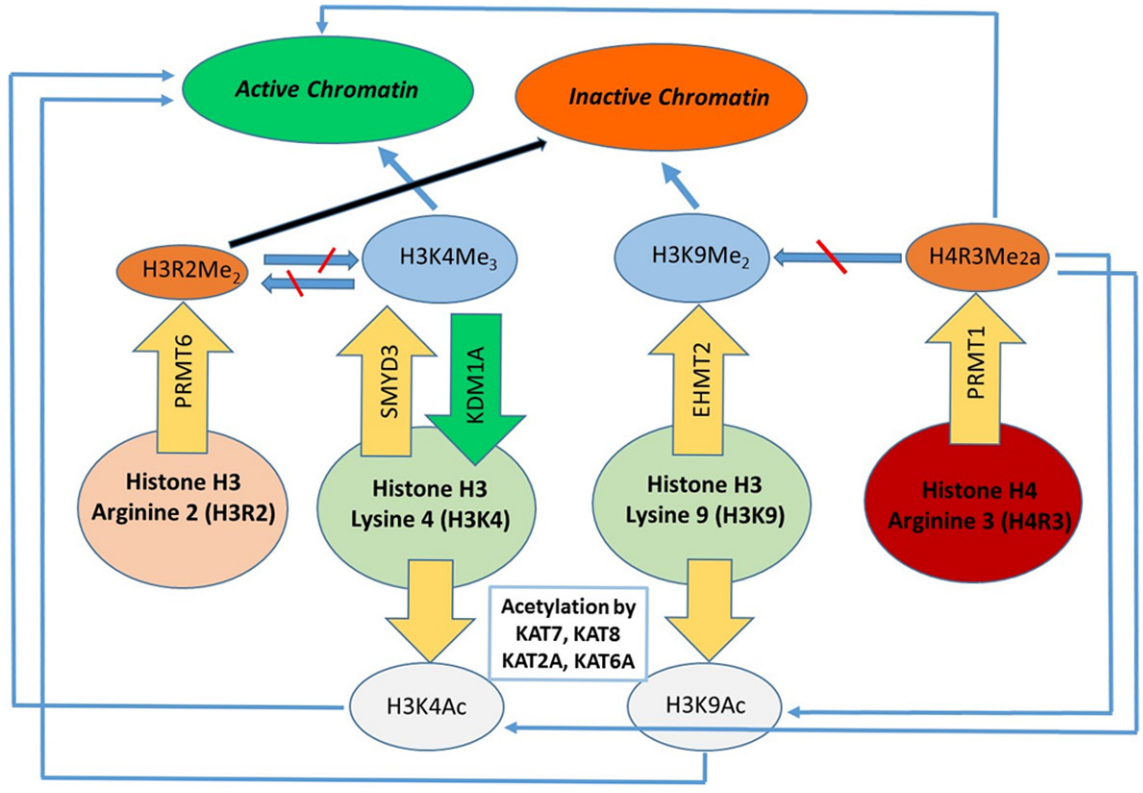

Figure 10. Schematic representation of shift of chromatin machinery towards more active state of chromatin in esophageal squamous cell carcinoma (ESCC) supported by findings of present study and available literature. The figure represents histone H3 amino acids residues Arginine 2 (H3R2), Lysine 4 (H3K4) and Lysine 9 (H3K9) and

Histone 4 residue Arginine 3 (H4R3). Methylation at H3R2, H3K4, H3K9 and H4R3 is catalyzed by PRMT6, SYMD3, EHMT2 and PRMT1 respectively. However, demethylation at H3K4 is catalyzed by KDM1A. Results show significantly higher expression of SMYD3, EHMT2 and PRMT1 as well as lower expression of KDM1A (not significant) in tumor tissue. PRMT6 is also found upregulated in tumor tissue (not significant), thus resulting in higher levels of H3R2, H3K4, H3K9 and H4R3 methylation. Mutual antagonism exists between H3K4 and H3R2 methylation states. Similarly active chromatin mark H4R3me2a (asymmetrical dimethylation) methylation catalyzed by PRMT1 is also reported to inhibit H3K9 methylation and facilitate acetylation at H3 Lysine residues mediated by KAT7, KAT8, KAT2A and KAT6A (all showed significant upregulation in present study). H3K4 methylation and H3 Lysine acetylation represents an active chromatin and H3K9 methylation is reported to be associated with an inactive chromatin state. In the present study upregulation of H4R3 methylation by PRMT1 might nullify the higher inactive state of chromatin mediated by increased H3K9 methylation. Active chromatin state is further enhanced by H3 Lysine acetylation and as a final point all this might signifies active state of chromatin leading to increased transcription in ESCC.

itation sequencing (ChIPSeq) analyses have also revealed the distribution of histone acetylation at promoters and enhancers and sometimes throughout the transcribed region of active genes [17, 18]. The significantly higher expression of histone acetyltransferases (KAT2A, KAT6A, KAT7 and KAT8) found in our data suggests more transcriptionally active chromatin state in tumor tissue compared to normal esophageal tissue.

Ash1L, DOT1L, EHMT2, SMYD3 are methyltransferases that showed higher expression in the present investigation. Absent, small, or homeotic discs 1-like (Ash1L) is the member of trithorax group of proteins. Both Ash1 and Ash1L located in promoter-proximal coding regions of a number of active genes, thereby suggesting their role in an early step of transcriptional elongation [19-21]. Both Ash1 and Ash1l possess histone lysine methyltransferase activity like other SET domain-containing proteins. Ash1lL methylates Lys36 of histone H3 to promote the establishment of Hox gene expression by counteracting Polycomb silencing. Ash1L-dependent Lys36 di-, tri-methylation of histone H3 in a coding region and exclusion of Polycomb group proteins occur independently of transcriptional elongation in embryonic stem (ES) cells as noted by Miyazaki and coworkers [22]. A novel regulatory cascade coordinated by Ash1L with RAR is suggested by the authors that provides insights into mechanisms underlying the establishment of the transcriptional activation that counteracts Polycomb silencing [22]. In a recent genome sequencing study ASH1L along with other histone regulatory genes are found to be frequently altered in ESCC [23]. All of the H3K36-specific methyltransferases, including ASH1L, HYPB, NSD1, and NSD2 are also reported to be inhibited by H2A monoubiquitination, whereas the other histone methyltransferases, including PRC2, G9a, and Pr-Set7 were not affected by this post-translation modification [24]. The findings collectively explain the mutual repulsion of H3K36me2/3 and Polycomb modifications [24]. Ash1L and other SET domain containing proteins (SETD1A and SMYD3) were up regulated in our study thus supporting the involvement of these proteins in the esophageal carcinogenesis.

DOT1L (DOT1-like, histone H3 methyltransferase) gene was found up regulated with marginal significance (p-value 0.05) in tumor which is an evolutionarily conserved histone methyltransferase that methylates lysine 79 of histone H3 (H3K79). It participates in the regulation of transcription, development, erythropoiesis, differentiation and proliferation. Kim and co-investigators reported that DOT1L siRNA-transfected lung cancer cell line displayed a non-proliferating multinucleated phenotype, abnormal mitotic spindle formation and centrosome number leading to chromosomal missegregation, cell cycle arrest at the G1 phase and induced senescence [25]. As over expression of a catalytically active DOT1L restored DOT1L siRNA-induced phenotypes, they suggested H3K79 methylation is a critical histone modification that regulates cell proliferation and is a novel histone mark for cancer [25]. Additionally, genome wide studies exhibit that H3K79 methylation is the marker of active transcription [26, 27]. Higher expression of DOT1L in tumor tissue of esophageal cancer found in present investigation is possibly a genetic marker of active transcription state of squamous esophageal cancer cells as suggested by the recent reports and warrant further validation.

Histone lysine methyltransferase EHMT2 (a key enzyme for histone H3 di-methylation at lysine-9) is localized in euchromatin regions and acts as a co-repressor for specific tran-

scription factors. Lu and co-workers reported that inhibition of EHMT2 decreases the overall H3K9Me2 level but not H3K27Me2 [28]. QRT-PCR and cDNA microarray analysis studies have found considerably elevated expression level of EHMT2 in bladder carcinomas and in different types of cancer [29]. Authors also found that the inhibition of EHMT2 subdued the growth and proliferation of cancer cells, and induced apoptosis by increasing caspase 8/caspase 3 activity thereby leading to inhibition of cell mobility and invasion. They also reported modulation of overall DNA methylation levels by EHMT2 inhibition in neuroblastoma cells [28]. Probably we are the first to report up regulation of EHMT2 in esophageal cancer that might lead to increased proliferation and reduced apoptosis to promote the ESCC.

The present investigation also reports higher expression of another histone methyltransferase (HMT) SET and MYND domain-containing protein 3 (SMYD3) gene that catalyzes histone H4 methylation at lysine 5 (H4K5me). H4K5me mark is also reported in different cell types and its formation is attenuated by depletion of Smyd3 protein [30]. SYMD proteins recently have also been shown to methylate non-histone proteins such as p53 and retinoblastoma tumor suppressor (pRb), and either protects their pro-apoptotic function or represses their apoptotic activity by methylation of different target lysine residues [31]. Another Locus-specific trimethylation of histone H3 lysine 4 (H3K4me) is also catalyzed by SMYD3 and reports suggest that H3K4me3 level defines a subset of hepatocellular carcinoma patients with distinct epigenetic phenotype and clinical outcome and suggested to be a novel predictor for poor prognosis [32].

Over expression of SMYD3 is reported to induce matrix metalloproteinase (MMP)-9 expression in transformed leukocytes and fibrosarcoma, which plays a central role in tumor progression and metastasis by stimulating cell migration, tumor invasion and angiogenesis [33]. The knockdown of SMYD3 by RNA interference (RNAi) decreased the level of H3K4me3 modification at MMP-9 promoter, reduced MMP-9 expression, and further reduced tumor cell proliferation [33]. Similarly, knockdown of SMYD3 in HeLa cell line by RNAi is also reported to inhibit cell growth and invasion [34]. Wang and coworkers suggest that a common VNTR polymorphism in the promoter region of SMYD3 gene might be a susceptibility factor for ESCC by interacting with tobacco carcinogens [35]. These findings revealed the role of SMYD3 in tumor progression and metastasis, and support our findings in ESCC in high risk northeast Indian population. SMYD3 and DOT1L both catalyze methylation at lysine residue and in combination can exert more profound effect on chromatin remodeling as both are found up-regulated in the present investigation. Lysine demethylases (KDM1A and KDM4A) were found down regulated and thus further strengthen the SMYD3 and DOT1L mediated effect on chromatin.

Lysine-specific demethylase 1(LSD1/KDM1A) is a H3K4 (histone H3 Lys4) demethylase and is reported to be associated with gene repression and is also found over expressed in different types of cancer. Jin and coworkers suggested that demethylating activity of LSD1 on p53 and DNA methyltransferase 1 is necessary for their stabilization. However, LSD1 is not found as an absolute requirement for their stabilization [36]. E-box-binding transcription repressors; Snail (SNAIL1) and Slug (SNAI2) are reported to be involved in the regulation of epithelial-mesenchymal transition (EMT) needed for the cancer cell invasion [37]. The report suggested that KDM1A interact with N-terminal SNAG domain of Snail to repress the expression of the EMT marker E-cadherin by epigenetic mechanisms [37]. Another study states that over expression of LSD1 is linked with poor prognosis in non-small cell lung cancer, and stimulated tumor cell proliferation, migration and invasion [38]. LSD1 is also found over expressed in human bladder carcinomas compared with non-neoplastic bladder tissues [39]. Although it is found 3.6 fold down regulated in present study, the result was not statistically significant. Still, a recent report proposed increased LSD1 expression in tumor tissue and found associated with lymph node metastasis and poorer overall survival in ESCC patients [40]. Further investigation warranted to explain this discrepancy as the present study was done in less number of samples.

Among arginine methyl transferase PRMT1, PRMT3, PRMT6 and PRMT8 all showed higher expression in tumor but only PRMT1 upregulation was found significant. PRMT1 catalyzes the asymmetrical dimethylation of the Arginine 3

residue in H4 (H4R3me2a) whereas PRMT5 catalyze the symmetrical dimethylation of the same residue (H4R3me2s) in vitro and in vivo. Studies have correlated H4R3me2s catalyzed by PRMT5 with transcriptional repression and H4R3me2a catalyzed by PRMT1 with transcriptional activation [41-43]. H4R3 methylation enables subsequent acetylation of H4 tails by p300 but acetylation of H4 inhibits its methylation by PRMT1 [43]. Down regulation of PRMT1 by siRNA in an erythroid cell line resulted in nearly complete loss of H4 Arg3 methylation across the chicken beta-globin domain. A domain-wide loss of histone acetylation on both histones H3 and H4, as well as an increase in H3 Lys9 (H3K9) and Lys27 (H3K27) methylation were reported. Methylated H3K9 and H3K27 marks were found to be associated with inactive chromatin. H4R3 methylation by PRMT1 was essential for the establishment or maintenance of a wide range of "active" chromatin modifications in vivo and in vitro [44]. In a recent study PRMT1 expression and asymmetric dimethylated modification of H4R3 catalyzed by PRMT1 were found up-regulated in glioma tissues and cell lines compared with normal brain tissues. Further down regulation of PRMT1 resulted in cellular arrest in the G1-S phase of the cell cycle, proliferation inhibition and apoptosis induction in glioma cell lines [45].

PRMT6 is reported as the mammalian methyltransferase for histone H3 at arginine 2 (H3R2) that catalyze asymmetric dimethylation of histone H3 at R2 (H3R2me2a) and its higher expression is found associated with suppression of motility and invasion by up-regulation of thrombospondin-1(a potent natural inhibitor of angiogenesis) and down-regulation of MMP-2 and 9 in breast and prostate cancer cells [46]. Wang and co-workers found that p16 arginine methylation is catalyzed by PRMT6 and reduced p16 arginine methylation level promotes the association of p16 with CDK4. The group also reported that PRMT6 over expression counteracts the cell cycle arrest at G1 phase induced by wild-type p16 in A549 cell line derived from adenocarcinomic human alveolar basal epithelial cells [42]. Similar reports reveal that knockdown of PRMT6 expression results in an accumulation of cells at the G2 checkpoint and induced senescence through upregulation of important cell cycle regulators, cyclin-dependent kinase (CDK) inhibitor gene p21 (p21 (CIP1/WAF1), CDKN1A), p16 (p16 (INK4A), CDKN2A) and p27 along with upregulation of well-known tumor suppressor p53 [47-49]. Phalke and co-workers have shown that PRMT6 acts as an oncogene in breast cancer cells that promotes growth and prevents senescence thus making it an attractive target for therapy [50].

PRMT6 is reported as a crucial negative regulator of H3K4 trimethylation and transcriptional activation [51]. However, another study reports that H3R2 methylation by PRMT6 was prevented by the presence of H3K4me3 on the H3 tail [52]. On the contrary, the H3R2me2a mark prevented methylation of H3K4 as well as binding to the H3 tail by an ASH2/WDR5/MLL-family methyltransferase complex [52]. The study concludes that mutual antagonism between H3R2 and H3K4 methylation, together with the association of MLL-family complexes with the basal transcription machinery, might contribute to the localized patterns of H3K4 tri-methylation characteristic of transcriptionally poised or active promoters in genome [52]. Another report shows that PRMT6 mediated H3R2 methylation decreases transcription levels of p53 and its targets, p21 and PML in mouse embryonic fibroblasts (MEFs). Further loss of enrichment of PRMT6 and H3R2 (me2a) repressive mark within the upstream region of p53 and an increase in the H3K4 (me3) activator mark in PRMT6 (-/-) MEFs was also suggested [49]. In the present investigation SMYD3 that catalyzes H3K4 methylation was found significantly upregulated. Although, PRMT6 was also showed higher expression, the result was not statistically significant. Therefore net global increase in H3K4 methylation in esophageal cancer due to significant SMYD3 upregulation might be correlated to transcriptionally active chromatin during carcinogenesis. These reports reveled that overexpression of PRMT1 and PRMT6 is a tumor promoting event as found in our experiments in esophageal tumor tissues compared to normal tissue. Although the result was not statistically significant in case of PRMT6. In the present study expression status of PRMT1 was found declined as we move from low grade well differentiated (Grade 1) to moderately differentiated (Grade 2) and further to high grade poorly differentiated (Grade 3) ESCC. Furthermore, this suggests the involvement of PRMT1 in the transition of low to high grade tumor formation. This is probably

the first report of involvement of PRMTs in esophageal cancer risk.

Among histone acetyl-transferases KAT7, KAT8, KAT6A and KAT2A were found upregulated in tumor tissue in present investigation. Histone acetyl-transferases transfer the acetyl moiety from acetyl co-enzyme A to lysine residues of histone proteins. Acetylation removes the positive charge on the histones, thereby decreasing the interaction of the N termini of histones with the negatively charged phosphate groups of DNA and transformed the condensed chromatin into a more relaxed structure [53]. This relaxed chromatin is reported to be associated with greater levels of gene transcription. Histones Lys acetylation interacts actively with other posttranslational modification agonistically or antagonistically [54]. HATs are classified into two classes namely type A and type B on the basis of cellular localization and substrate specificity. Type A are specific for nucleus that acetylates histones present on the chromatin and have widespread role in nuclear processes. However, type B are found partially localized in the cytoplasm and specific for free histone substrates [55]. Whereas, histone deacetylases (HDACs) remove the acetyl groups reestablishing the positive charge in the proteins [56]. KAT7 (Lysine acetyltransferase 7) is previously known as histone acetyltransferase (HAT) Hbo1 which is unique among HAT enzymes because it serves as a positive regulator of DNA replication, and Hbo1 protein is reported to be highly expressed in esophageal cancer [57]. HBO1 acetylates lysine residues of histones and JADE1 binds to HBO1 thus promoting acetylation of histones in chromatin context. The binding of JADE1 in the HBO1-HAT complex is needed for chromatin recruitment of replication factors and gene expression during cell cycling in cultured epithelial cells [58].

Five members of the ING (inhibitor of growth) family of growth regulators are reported in human (ING1 to ING5) and divided into three groups (ING1/2, ING3, and ING4/5) based on their association with three distinct types of protein complexes that regulate chromatin modification and structure via histone acetylation and deacetylation [59]. These complexes that carry out histone acetylation contain members of the MYST family of HATs that include Tip60 (KAT5), HBO1 (KAT7), MOZ (KAT6A), MORF (KAT6B) and MOF (KAT8) as their catalytic subunits [59]. One of the ING catalytic subunits KAT2A (K(lysine) acetyltransferase 2A) or GCN5, is a HAT that functions primarily as a transcriptional activator and is also reported to function as a repressor of NF-kappa-B by promoting ubiquitination of the NF-kappa-B subunit RELA in a HAT-independent manner [60]. KAT8 protein expression status was found declined from low grade well differentiated (Grade 1) to moderately differentiated (Grade 2) and further to high grade poorly differentiated (Grade 3) ESCC in the present investigation. This suggests the involvement of KAT8 in the transition of low to high grade tumor formation in ESCC. Similar trends were also found for KAT2A but the result was not found statistically significant. The increased expression of KAT7, KAT8, KAT6A and KAT2A in ESCC tissues found in the present investigation could be one of the important factors for the assessment of role of chromatin modification in esophageal carcinogenesis.

On the contrary histone deacetylases HDAC11 and HDAC9 were also found highly expressed in tumor tissues in the present investigation. HDAC11 is a zinc-dependent HDAC of class IV and is reported to be overexpressed in several carcinomas as compared to corresponding normal tissues [61]. HDACs have emerged as effective therapeutic targets for cancer as these enzymes are directly involved in the gene regulation through chromatin modification. Signal transduction mediators, transcription factors and regulators, DNA repair enzymes, chaperon protein, inflammation mediators are some other reported non-histone targets of HDACs and their acetylation can affect various cellular pathways including control of gene expression, regulation of cell proliferation, cell death and angiogenesis [62]. Histone deacetylase enzyme HDAC11 expression was found higher in tumor tissue but was not statistically significant, however HDAC9 upregulation of expression was found marginally significant (p-value 0.053).

Aurora family kinases (AURKA, AURKB and AURKC) are reported to be involved in formation of a bipolar mitotic spindle, segregation of chromosomes and the completion of cytokinesis. As frequently deregulated in cancer and able to transform cells in vitro they are considered to be attractive drug targets [63]. NIMA is another gene found to be related to cell cycle

regulation. NIMA (never in mitosis gene a)-related kinase 6 (NEK6) is a serine/threonine kinase that belongs to the Neks (NIMA-related kinases) family, which has been involved in mitosis control [64]. Kasap and co-investigators reported significantly higher levels of AURKA, AURKB and NEK6 in esophageal adenocarcinoma compared to the control group in a study done in biopsies collected from esophageal adenocarcinoma, erosive esophagitis and normal esophagous. As well as AURKA, AURKC, HDAC9 and NEK6 were found to be expressed at significantly higher levels in erosive esophagitis compared to the control group [64]. Similarly, other report states that NEK6 is overexpressed in hepatocellular carcinoma as compared with the adjacent normal tissue along with higher expression of NEK6 is found to be associated with histological grade and poor prognosis [65]. The result of the present investigation also suggests AURKC and NEK6 as over expressed chromatin modifiers in ESCC.

Serine/threonine protein kinase p90-kDa ribosomal S6 kinase (RSK) is an important downstream effector of MAPK and its inhibition resulted in decreased proliferation of the human prostate cancer lines [66]. Enzastaurin, a protein kinase C beta inhibitor induces apoptosis through RSK-mediated and Bad-mediated pathways, besides inhibiting the Akt signal cascade in gastric cancer cells as noted by Lee and co-investigators [67]. The authors also found synergistic effects of enzastaurin when combined with other drugs such as 5-fluorouracil, cisplatin, paclitaxel, or irinotecan. RSK2 amplification is frequently found in gastric cancer and is related to a poor prognosis in a recent report [68]. Another report on breast cancer describes that phosphorylation of YB-1 at the serine 102 residue is mediated by RSK1/RSK2 and is required for transcriptional activation of growth-enhancing genes, such as EGFR. This implicates the EGFR/RSK/YB-1 pathway as an important component in Basal-like breast cancers [69]. Present investigation for the first time reports the increased expression of Ribosomal protein S6 kinase polypeptide 5 in esophageal cancer. As its role in gastric and breast cancer this could be further explored as a drug target in esophageal cancer.

The study concludes that increased expression of histone methylation and acetylation catalyzing enzymes, and decreased expression of demethylases are associated with ESCC. As both histone acetyltransferase and deacetylases showed over expression and this seems to be contradictory and might be a limitation of the study because it has been done in a limited sample size. Probably the situation might be clear after repeating the study in higher number of sample size. The literature of changes catalyzed by these enzymes at specific histone protein residues are available but the studies that revealed the genome wide localization of these changes in esophageal carcinogenesis are not yet reported and needs to be done. We have hypothesized a complex interplay of PRMT1, PRMT6, SMYD3, EHMT2, KDM1A, KAT7, KAT8, KAT2A and KAT6A mediated histone modifications with the help of available literature [18, 52, 70] and the findings of present study that suggests manifestation of an enhanced active chromatin state in esophageal carcinogenesis (**Figure 10**).

Reports suggest lifestyle related factors such as dietary, smoking, alcohol consumption, behavior, stress, physical activity and working habits might also influence epigenetic mechanisms such as DNA methylation, histone acetylation and microRNA expression [71]. In the present study cigarette smoking was found as a risk factor in the studied subjects although the result was not statistically significant. A study done in rat animal model reveals that the acetylation of H4 and phospho-acetylation of H3 significantly increased in lung tissue after cigarette smoke exposure [72]. Histone acetyltransferases were found upregulated in our study therefore it can be hypothesized that cigarette smoking habit could results in elevated H4 acetylation in the studied subjects by means of modulation of the expression of acetyltransferases by some unknown mechanisms. Finally the study opens a window to start with some of these chromatin modifiers and explore the genome wide characterization of the histone changes driven by them through ChiP-Seq experiments in ESCC. Further comparison of these genome wide histone posttranslational changes with global gene expression would be needed to find out epigenetically regulated genes. Along with that the effect of altered expression of these genes on cell proliferation, invasion and drug efficacy needed for better diagnosis and treatment of patients and to discover epigenetic markers for ESCC.

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Disclosure of conflict of interest

None.

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