Indian J Med Res 150, December 2019, pp 584-591
DOI: 10.4103/ijmr.IJMR_620_18

Quick Response Code:

Promoter-associated DNA methylation & expression profiling of genes (*FLT 3, EPB41L3* & *SFN*) in patients with oral squamous cell carcinoma in the *Khasi* & *Jaintia* population of Meghalaya, India

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Received March 28, 2018

***Background & objectives*: Oral squamous cell carcinoma is one of the most lethal forms of cancer, and its aetiology has been attributed to both genetic and epigenetic factors working in liaison to contribute to the disease. Epigenetic changes especially DNA methylation is involved in the activation or repression of gene functions. The aim of this study was to investigate the DNA methylation pattern and expression profiling of the promoter regions of FMS-related tyrosine kinase 3 (*FLT3*), erythrocyte membrane protein band 4.1-like 3 (*EPB41L3*) and stratifin (*SFN*) genes in oral cancer within the *Khasi* and *Jaintia* tribal population of Meghalaya in North East India.**

***Methods*: Quantitative methylation analyses of the selected genes were carried out by MassARRAY platform System, and the relative expression profiling was carried out by real-time polymerase chain reaction.**

***Results*: Quantitative methylation results indicated that the level of methylation was significantly higher (hypermethylated) for *FLT3* and *EPB41L3* and significantly lower (hypomethylated) for *SFN* in tumour tissues as compared to the adjacent paired normal tissue. Expression profiling was in concurrence with the methylation data whereby hypermethylated genes showed low mRNA level and vice versa for the hypomethylated gene.**

***Interpretation & conclusions*: The findings show that hyper- and hypomethylation of the selected genes play a potential role in oral carcinogenesis in the selected *Khasi* and *Jaintia* tribal population of Meghalaya. The methylation status of these genes has not been reported in oral cancer, so these genes may serve as promising biomarkers for oral cancer diagnosis as well as in disease monitoring.**

Key words DNA methylation - ethnic population - gene expression - hypermethylation - hypomethylation - oral cancer

Oral squamous cell carcinoma (OSCC) or oral cancer is the sixth most common form[1] and one of the most widespread and highly aggressive types of cancer[2]. It has an annual estimated incidence of over 500,000 cases worldwide[1], and in India, as per the Global Cancer Statistics 2018 the incidence of new cases of cancer of the lip and oral cavity is about 119,992[3]. A case study carried out in India in 2010 on

cancer mortality rate revealed that the cancer of the oral cavity was the third most common cancer among women, with the highest incidence of mortality among men. The study also showed that the highest incidence of cancer occur mainly in the northeastern parts of India[4]. As per the Cancer Atlas of India, Meghalaya has a high rate of cancer of the oral cavity, oropharynx, hypopharynx and oesophageal cancer[5]. In Meghalaya, a retrospective study carried out in Shillong Civil Hospital from 2007 to 2011 on the prevalence of head and neck cancer within the State showed that oropharyngeal and oral cancer were the most common in this region[6].

The high rate of oral cancer in Meghalaya has been attributed to the lifestyle habits of the population. The involvement of betel quid has been shown in several types of cancer including oral cancer[7,8].

The development of oral cancer involves many factors working together in synergy to bring about alterations in two large groups of genes, *viz*., oncogenes and tumour suppressor genes (TSGs). These alterations bring about molecular changes in both genetic and epigenetic levels leading to the diseased condition[9]. Epigenetic modifications such as DNA methylation (hyper- and hypomethylation) of a specific gene(s) is known to be one of the factors involved in the development and progression of oral cancer[10]. DNA hypermethylation of promoter region(s) of specific TSGs is a critical step in oral carcinogenesis[11], and several genes hypermethylated in OSCC are known to cover a wide range of cellular processes[2]. Global genomic hypomethylation of repetitive DNA elements and oncogenes is a common feature in many malignancies, and their activation has been known to trigger genomic instability[12].

A whole epigenomic study carried out by us revealed 45 differentially methylated genes[13], and the present study was focused on the study of the methylation pattern of three selected genes, *viz*., FMS-related tyrosine kinase 3 (*FLT3*), erythrocyte membrane protein band 4.1-like 3 (*EPB41L3*) and stratifin (*SFN*) from those 45 genes, in patients with oral cancer in the *Khasi* and *Jaintia* tribal population of Meghalaya, India. Several studies have shown a disparity in the level of DNA methylation of specific functional gene and their altered expression based on race and ethnicity[14-16], the present study may provide an insight on how promoter-associated CpG island methylation of specific functional genes is involved in the aetiology of oral cancer within this population.

Material & Methods

Surgically resected tumour and adjacent paired normal samples (at least 2 cm from the site of the primary tumour) were collected from the department of ENT, North Eastern Indira Gandhi Regional Institute of Health and Medical Sciences (NEIGRIHMS), Shillong, Meghalaya, India, from April 2013 to February 2014. Samples were collected from the ethnic tribal patients of Meghalaya *i.e*., the *Khasis* and *Jaintias*. The samples were flash-frozen in liquid nitrogen and stored at −80˚C until further requirement. The tumour tissues were histopathologically confirmed for cancer cells. All participants provided written informed consent. The research protocol was approved by the Institutional Ethics Committee of North Eastern Hill University, Shillong, Meghalaya (IEC/13412).

DNA extraction and MassARRAY: A total of 17 pairs of tumour and paired adjacent normal tissues were used for methylation profiling by MassARRAY. Quantitative methylation MassArray EpiTyper (EpiTYPER® Agena Bioscience, CA, USA)[13] was carried out for the selected genes, *viz., FLT3, EPB41L3* and *SFN* for methylation analysis of its promoter region-associated CpG island. This bisulphite treatment-based method for detection and quantitation of DNA methylation uses base-specific cleavage and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to differentiate between methylated and non-methylated DNA. All primers were designed using MethPrimer[17] within the identified CpG islands (Table I). The details of procedure have been described elsewhere[13].

RNA extraction and RT-PCR: RT-PCR was carried out for all three genes (*FLT3, EPB41L3* and *SFN*) using β-actin as an endogenous control. Total RNA was extracted from tumour and paired adjacent normal tissues as described eleswhere[13]. mRNA sequence for the selected genes was retrieved from PubMed gene database (*https://www.ncbi.nlm.nih.gov/pubmed/*), and gene-specific primers were designed using Primer3web v4.0.0 (*http://primer3.ut.ee/*)[1] and synthesized by Integrated DNA Technologies, Iowa, USA (Table II). Quantitative RT-PCR was performed and relative expression was calculated using the comparative delta Ct method[13].

Statistical analysis: Kolmogorov-Smirnov test was performed over the raw data of methylation and expression assay to check whether the data are normally

Table I. List of primers for the selected sets of genes used in the MassARRAY® EpiTYPER

Amplicon name		Primer sequence	Amplicon size (bp)	Amplicon location relative to TSS	Total number of CpGs
FLT3_CpG_1	F	TATTTTTAAGAGAGTTATTTGTAG	541	−37 to +503 bp	75
	R	AAAAAAAATCCTTAACCACCTAAC			
EPB41L3_CpG_1	F	TTTATGTAATTGTTTTGAAGTATTG	288	−199 to +88 bp	32
	R	TTACCTAAAATCAACAAAAAACCC			
SFN_CpG_2	F	GGATATGGTAGTTTTTATGAAAGG	259	+111 to +369 bp	15
	R	ATAACTATCCAACAAACCCAACAC			

TSS, transcription start site; FLT3, FMS-related tyrosine kinase 3; EPB41L3, erythrocyte membrane protein band 4.1-like 3; SFN, stratifin; bp, base pair; F, forward; R, reverse

Table II. List of primers for quantitative real-time polymerase chain reaction

Gene		Primer sequence	Amplicon size (bp)
EPB41L3 (NM_012307.3)	F	AGCAGTAAACTCTCTCGGTCT	112
	R	AGCGTTTCTCTACATCACAGG	
FLT3 (NM_004119.2)	F	TCAGTGGCAAGAAACGACAC	90
	R	TCCCTTTTCTACGATGGTAACC	
SFN (NM_006142.3)	F	TTGTGGCTGAGAACTGGACA	113
	R	TGCTTTCCCTCAATCTCGGT	
ACTB (NM_001101)	F	TCTACAATGAGCTGCGTGTG	110
	R	GGTCTCAAACATGATCTGGGT	

ACTB, β-actin

distributed or not. Data were not normally distributed and hence required a non-parametric statistical test. Taking into consideration the small sample size, the result of normal distribution and paired nature of the sample, the Wilcoxon signed rank test for paired analysis was chosen for data analysis. All statistical analysis was carried out in SPSS v20.0 (IBM Corp., Armonk, NY, USA).

Results

Methylation level of FLT3, EPB41L3 and SFN: MassARRAY platform was used for the methylation analysis of *FLT3, EPB41L3* and *SFN*. Quantitative methylation analyses were primarily focused on the promoter region of the gene. Hence, one amplicon for each genes was identified taking into consideration SNPs, repeats, a maximum number of CpGs and their relative position with respect to the transcription start site (TSS).

The methylation level of the each amplicon for the three genes showed a significant difference in the average level of methylation in tumour samples as compared to that of adjacent normal tissue. *FLT3* amplicon with 75 CpG sites analysed showed a significantly higher level of average methylation across 46 CpG sites (P <0.05, P<0.01 and P<0.001, n=17) in tumour sample as compared to that of adjacent paired normal tissue (Fig. 1). Twenty three sites could not be detected and six sites were not significant. Similarly, *EPB41L3* amplicon with 32 CpG sites showed a significantly higher level of average methylation across 18 CpG sites (P<0.01 and P<0.001, n=17) in tumour sample as compared to that of adjacent paired normal tissues (Fig. 2). Fourteen sites could not be detected. The higher level of DNA methylation in tumour sample as compared to that of paired adjacent normal tissue gives indicated that the CpGs within the amplicon of *FLT3* and *EPB41L3* were hypermethylated.

In the case of SFN, of the 15 CpG sites analysed, the level of average methylation across 10 CpG sites was significantly lower in tumour sample as compared to that of adjacent paired normal tissues (P<0.05, n=17). Two sites could not be detected and three sites were NS (Fig. 3). This suggested that CpGs within the amplicon of SFN were hypomethylated.

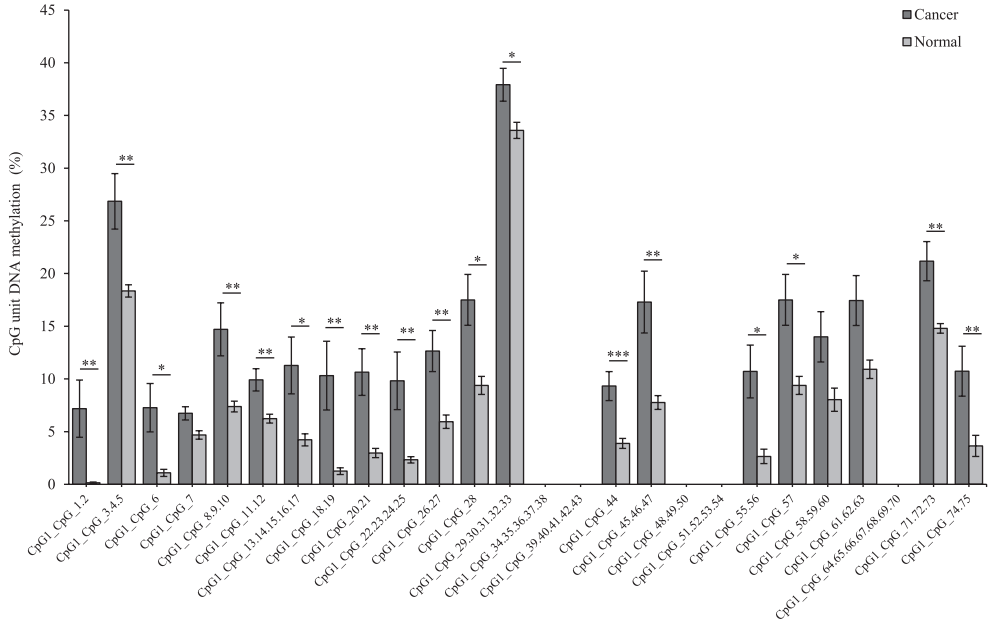

Fig. 1. Average methylation level of each CpG site in FMS related tyrosine kinase 3 (*FLT3*) in tumour and adjacent normal tissues (n=17) detected and undetected by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis. P^{*}<0.05, **<0.01, ***<0.001 compared to normal. Data are shown as mean±standard error of mean.

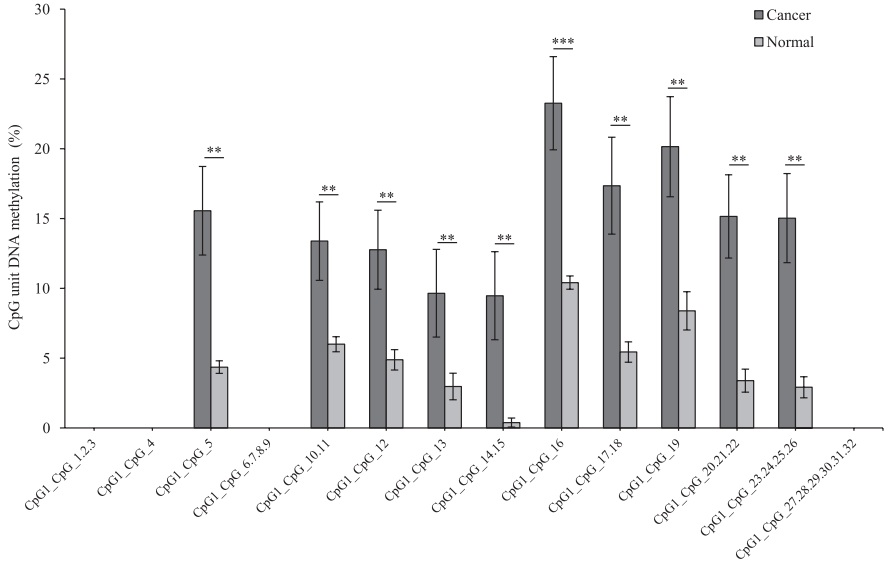

Fig. 2. Average methylation level of each CpG site in erythrocyte membrane protein band 4.1-like 3 (*EPB41L3*) in tumour and adjacent normal tissues (n=17) detected and not detected by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis. P^{*}<0.05, **<0.01, ***<0.001 compared to normal. Data are shown as mean±standard error of mean.

Expression analysis by RT-PCR: Quantitative real-time PCR of *FLT3, EPB41L3* and *SFN* were carried out on 12 paired samples. The expression of *FLT3* and *EPB41L3* showed a significant reduction in the level of mRNA expression in tumour sample as compared to that of adjacent paired normal tissues (P<0.01). There was an approximate 6-fold and 4-fold decrease in the level of mRNA expression relative to *FLT3* and *EPB41L3,* respectively (Fig. 4). SFN, on the other hand, showed a significant increase in the level of mRNA expression in the tumour sample as compared to that of adjacent paired normal tissues (P<0.01, n=12). A 21-fold increase in the level of mRNA expression relative to SFN was observed. The level of mRNA expression seen in *FLT3* and *EPB41L3* was in conjunction with the methylation data as hypermethylation usually

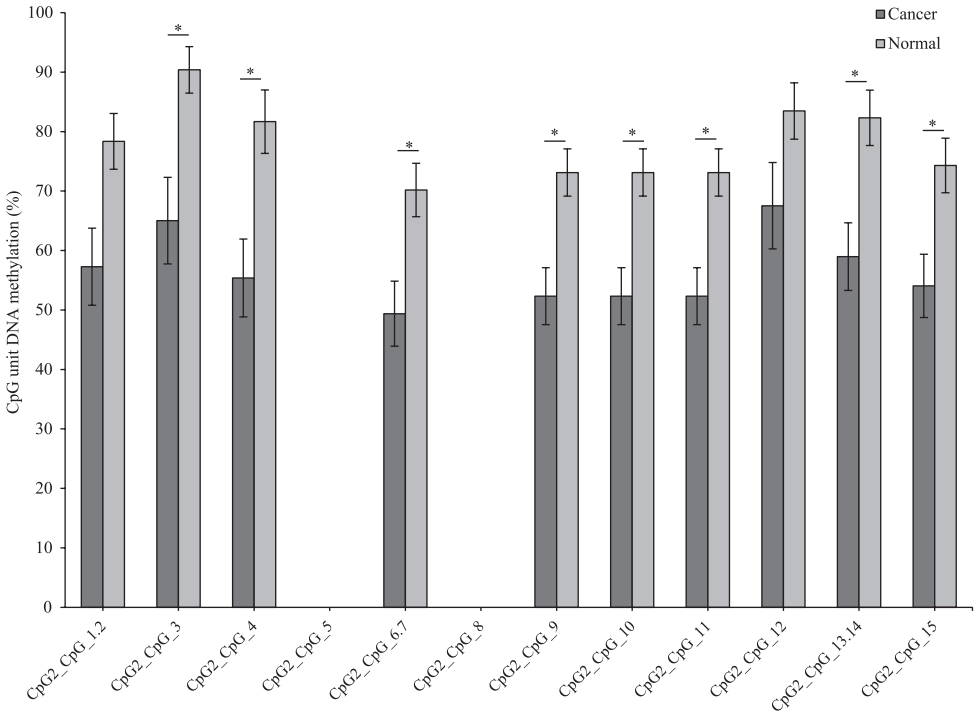

Fig. 3. Average methylation level of each CpG site in stratifin (*SFN*) in tumour and adjacent normal tissues (n=17) detected and undetected by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis. $^{*}P<0.05$ compared to normal. Data are shown as mean±standard error of mean.

decreases the level of expression. Similarly, *SFN* level of mRNA expression was in concurrence with the methylation data as hypomethylation usually increases the level of expression (Fig. 4).

Discussion

Promoter hyper- or hypomethylation for genes, especially around the TSS has been known to play a critical role in cancer development, especially at an early stage of tumour development[13]. These early alterations lead to either loss of function of TSGs or gain in function of oncogenic genes and elements. Altered gene function results in loss of cell cycle control, up- or downregulation of gene transcription and altered cell-cell and cell-substratum interaction. The three genes (*FLT3, EPB41L3* and *SFN*) showed their role with reference to cell function, and alteration in their functionality by methylation pattern may give rise to altered gene expressions and abnormal development.

FLT3 belongs to the receptor tyrosine kinase family and is important for the normal development of the haematopoietic and immune systems[18]. Many studies on *FLT3* have been focused on the somatic alteration of the gene consisting of an internal tandem duplication of the JM (juxtamembrane) domain coding sequence in acute myeloid leukemia[19]. Studies carried out on the status of DNA methylation profiling of *FLT3* gene and cancer showed a higher level of DNA methylation within the *FLT3* gene in oesophageal cancer[20] and pancreatic cancer[21], respectively, indicating that the gene was hypermethylated. Tan *et al*[21] reported a decrease in the level of mRNA expression in pancreatic cancer[2], and treatment on HCC cell lines by 5-aza-dC showed re-expression of the methylated genes[22]. Underexpression of the *FLT3* genes may contribute to the lack of sensitivity in certain tyrosine kinase target drugs, such as imatinib[23]. Kuo *et al*[20] have reported that *FLT3* may function as tumour suppressor-like genes in oesophageal squamous cell carcinoma (ESCC) tumourigenesis as hypermethylation of the gene showed an association with poor survival outcome. No literature was available for the methylation status of this gene in oral cancer.

The *EPB41L3* gene encodes for an adhesion protein belonging to the 4.1 family of membrane-associated proteins that regulates cell growth[9] and cell adhesion[24]. *EPB41L3* promoter hypermethylation has been attributed to the cause of ovarian cancer[25], cervical cancer[24], diffuse gliomas[9] and prostate cancer[26]. Hypermethylation of the *EPB41L3* promoter is a common mechanism

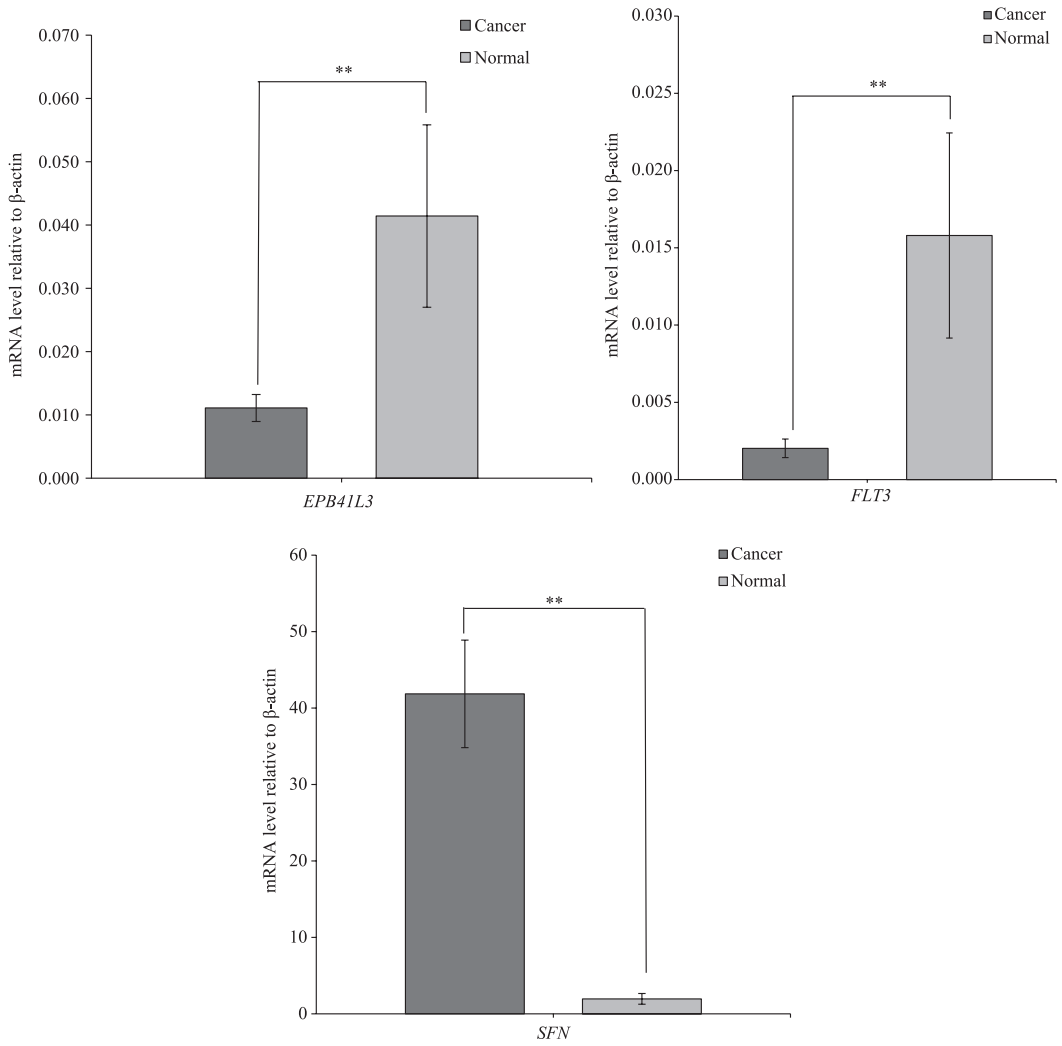

Fig. 4. Average mRNA expression of FMS-related tyrosine kinase 3 (*FLT3*), erythrocyte membrane protein band 4.1 like-3 (*EBP41L3*) and stratifin (*SFN*). Real-time polymerase chain reaction was performed to identify the mRNA expression of all three genes in tumour and adjacent paired normal tissues (n=12). $^{**}P<0.01$ compared to normal. Data are shown as mean±standard error of mean.

by which the gene is downregulated during tumour development and re-expression of *EPB41L3* has been known to induce extensive apoptotic cell death[25]. *EPB41L3* may play an important role in suppressing metastasis by regulating the proper arrangements of actin stress fibres and increasing the cell motility associated with metastatic behaviour[27]. Because of this feature, *EPB41L3* may be considered as a tumour suppressor in ESCC[27] and a candidate ovarian cancer-suppressor gene[25]. A study carried out by Perez-Janices *et al*[9], showed that the expression of *EPB41L3* in gliomas was low or completely absent in cases where the promoter region of the gene was hypermethylated. Several studies also showed that treatment of cell lines by 5-azadC restored transcription of the *EPB41L3* gene[9,24].

SFN belongs to the 14-3-3 family of acidic polypeptides. Capable of binding to more than 100 functionally diverse cellular proteins, these can play significant roles in different cellular functions such as cell cycle regulation, signal transduction, apoptosis, malignant transformation and cytoskeleton organization[28]. Overexpression of *SFN* has been reported in lung cancer[29] and pancreatic cancer[21]. The overexpression of the *SFN* gene has been attributed to hypomethylation, as confirmed by Sato *et al*[30] in pancreatic cancer.

Our study results were in concurrence with other published studies whereby promoter hyper- and hypomethylation of the functional genes in OSCC were shown to lead to their altered gene expressions. Our study also showed involvement of new and novel

genes involved in OSCC, and this might further provide an additional insight on the existing molecular mechanism involved in the occurrence, development and progression of the disease.

***Acknowledgment*:** Authors acknowledge Shri Shaiphyrnai Marnagar (Project Assisstant-DBT project) for providing support in laboratory maintenance, Dr Prashant Singh and the team at the Genomics Shared Resources, Rosewell Park Cancer Institute, Buffalo, New York, for providing all the technical support required for carrying out the MassArray EpiTyper experiment.

***Financial support & sponsorship*:** Authors acknowledge the funding support provided by the Department of Biotechnology (DBT), New Delhi, in the form of financial support (BT/PR5546/MED/12/560/2012 dated 09/09/2013 awarded to the last author (SG) and Council of Scientific & Industrial Research (CSIR), New Delhi, for providing Senior Research Fellowship (CSIR-SRF) to the first author (SK).

***Conflicts of Interest*:** None.

References

1. Lingen M, Pinto A, Mendes R, Franchini R, Czerninski R, Tilakaratne W, *et al.* Genetics/epigenetics of oral premalignancy: Current status and future research. *Oral Dis* 2011; *17* : 7-22.
2. Mascolo M, Siano M, Ilardi G, Russo D, Merolla F, de Rosa G, *et al.* Epigenetic disregulation in oral cancer. *Int J Mol Sci* 2012; *13* : 2331-53.
3. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; *68* : 394-424.
4. Dikshit R, Gupta PC, Ramasundarahettige C, Gajalakshmi V, Aleksandrowicz L, Badwe R, *et al.* Cancer mortality in India: A nationally representative survey. *Lancet* 2012; *379* : 1807-16.
5. Cancer Atlas India. Available from*: http://www.canceratlasindia.org/*, accessed on December 12, 2017.
6. Shunyu NB, Syiemlieh J. Prevalence of Head and Neck Cancer in the State of Meghalaya : Hospital-based Study. *Int J Head Neck Surg* 2013; *4* : 1-5.
7. Wary KK, Sharan RN. Aqueous extract of betel-nut of North-East India induces DNA-strand breaks and enhances rate of cell proliferation *in vitro*. *J Cancer Res Clin Oncol* 1988; *114* : 579-82.
8. Gasche J, Goel A. Epigenetic mechanisms in oral carcinogenesis. *Futur Oncol* 2013; *8* : 1407-25.
9. Perez-Janices N, Blanco-Luquin I, Tuñón MT, Barba-Ramos E, Ibáñez B, Zazpe-Cenoz I, *et al. EPB41L3*, *TSP-1* and *RASSF2* as new clinically relevant prognostic biomarkers in diffuse gliomas. *Oncotarget* 2015; *6* : 368-80.
10. Ogi K, Toyota M, Ohe-Toyota M, Tanaka N, Noguchi M, Sonoda T, *et al.* Aberrant methylation of multiple genes and clinicopathological features in oral squamous cell carcinoma. *Clin Cancer Res* 2002; *8* : 3164-71.
11. Viet CT, Schmidt BL. Methylation array analysis of preoperative and postoperative saliva DNA in oral cancer patients. *Cancer Epidemiol Biomarkers Prev* 2008; *17* : 3603-11.
12. Ting Hsiung D, Marsit CJ, Houseman EA, Eddy K, Furniss CS, McClean MD, *et al.* Global DNA-methylation level in whole blood as a biomarker in head and neck squamous cell carcinoma. *Cancer Epidemiol Biomarkers Prev* 2007; *16* : 108-14.
13. Khongsti S, Lamare FA, Shunyu NB, Ghosh S, Maitra A, Ghosh S. Whole genome DNA methylation profiling of oral cancer in ethnic population of Meghalaya, North East India reveals novel genes. *Genomics* 2017; *2* : 112-23.
14. Piyathilake CJ, Bell WC, Jones J, Henao OL, Heimburger DC, Niveleau A, *et al.* Pattern of nonspecific (or global) DNA methylation in oral carcinogenesis. *Head Neck* 2005; *27* : 1061-7.
15. Song MA, Brasky TM, Marian C, Weng DY, Taslim C, Dumitrescu RG, *et al.* Racial differences in genome-wide *methy*lation profiling and gene expression in breast tissues from healthy w*omen. Epigenetics* 2015; *10* : 1177-87.
16. Zhang FF, Cardarelli R, Carroll J, Fulda KG, Kaur M, Gonzalez K, *et al.* Significant differences in global genomic DNA methylation by gender and race/ethnicity in peripheral blood. *Epigenetics* 2011; *6* : 623-9.
17. Li LC, Dahiya R. MethPrimer: designing primers for methylation PCRs. *Bioinformatics* 2002; *18* : 1427-31.
18. Levis M, Small D. *FLT3*: ITDoes matter in leukemia. *Leukemia* 2003; *17* : 1738-52.
19. Noguera NI, Brecci*a* M, Divona M, Diverio D, Costa V, De Santis S, *et al.* Alterations of the *FLT3* gene in acute promyelocytic leukemia: Association with diagnostic characteristics and analysis of clinical outcome in patients treated with the Italian AIDA protocol. *Leukemia* 2002; *16* : 2185-9.
20. Kuo IY, Chang JM, Jiang SS, Chen CH, Chang IS, Sheu BS, *et al.* Prognostic CpG methylation biomarkers identified by methylation array in esophageal squamous cell carcinoma patients. *Int J Med Sci* 2014; *11* : 779-87.
21. Tan AC, Jimeno A, Lin SH, Wheelhouse J, Chan F, Solomon A, *et al.* Characterizing DNA methylation patterns in pancreatic cancer genome. *Mol Oncol* 2009; *3* : 425-38.
22. Shin SH, Kim BH, Jang JJ, Suh KS, Kang GH. Identification of novel methylation markers in hepatocellular carcinoma using a methylation array. *J Korean Med Sci* 2010; *25* : 1152-9.
23. Chen YC, Huang RL, Huang YK, Liao YP, Su PH, Wang HC, *et al.* Methylomics analysis identifies epigenetically silenced genes and implies an activation of β-catenin signaling in cervical cancer. *Int J Cancer* 2014; *135* : 117-27.
24. Guerrero-Setas D, Pérez-Janices N, Blanco-Fernandez L, Ojer A, Cambra K, Berdasco M, *et al.* RASSF2 hypermethylation is present and related to shorter survival in squamous cervical cancer. *Mod Pathol* 2013; *26* : 1111-22.
25. Dafou D, Grun B, Sinclair J, Lawrenson K, Benjamin EC, Hogdall E, *et al.* Microcell-mediated chromosome transfer identifies *EPB41L3* as a functional suppressor of epithelial ovarian cancers. Neoplasia Neoplasia Press, Inc.; 2010; *12* : 579-89.

26. Schulz WA, Alexa A, Jung V, Hader C, Hoffmann MJ, Yamanaka M, *et al*. Factor interaction analysis for chromosome 8 and DNA methylation alterations highlights innate immune response suppression and cytoskeletal changes in prostate cancer. *Mol Cancer* 2007; *6* : 1-16.

27. Zeng R, Huang JP, Li XF, Xiong WB, Wu G, Jiang ZJ, *et al. Epb41l3* suppresses esophageal squamous cell carcinoma invasion and inhibits MMP2 and MMP9 expression. *Cell Biochem Funct* 2016; *34* : 133-41.

28. Shiba-Ishii A, Noguchi M. Aberrant stratifin overexpression is regulated by tumor-associated CpG demethylation in lung adenocarcinoma. *Am J Pathol* 2012; *180* : 1653-62.

29. Shiba-Ishii A, Kano J, Morishita Y, Sato Y, Minami Y, Noguchi M. High expression of stratifin is a universal abnormality during the course of malignant progression of early-stage lung adenocarcinoma. *Int J Cancer* 2011; *129* : 2445-53.

30. Sato N, Maitra A, Fukushima N, van Heek NT, Matsubayashi H, Iacobuzio-Donahue CA, *et al.* Frequent hypomethylation of multiple genes overexpressed in pancreatic ductal adenocarcinoma frequent hypomethylation of multiple genes overexpressed in pancreatic. *Cancer Res* 2003; *63* : 4158-66.

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