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Key Words:	methylation, EDNRB , KIF1A, OSCC, p16INK4a, DCC, nodal metastasis

Promoter Hypermethylation in Indian Primary Oral Squamous Cell Carcinoma

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Short Title: Aberrant methylation in Indian oral SCC

Key Words: methylation, EDNRB, KIF1A, OSCC, $p16^{INK4a}$, DCC, nodal metastasis

Abbreviations:

DCC: Deleted in Colorectal Carcinoma; *EDNRB*: Endothelin receptor type B; *KIF1A*: Kinesin chain member 1A, HNSCC: Head and neck squamous-cell carcinoma; OSCC: Oral Squamous Cell Carcinoma; Q-MSP: quantitative methylation-specific PCR; CDK: Cyclin Dependent Kinase; ET: Endothelin

Statements describing novelty and impact of this paper:

Development of DNA methylation based non-invasive biomarkers offer the possibility of using rapid diagnostic assays for cancer screening and diagnosis. In a recent study, some of us (JC, SD) have demonstrated the diagnostic utility of analysis of promoter methylation of a panel of genes in HNSCC in North American population. In view of the differences in molecular alterations in HNSCC related to geographic location, perhaps due to differences in carcinogen exposure, it is important to compare the performance of this panel of genes in other populations including the South-East Asian population. This study demonstrates the utility of promoter hypermethylation of this panel of four genes – Deleted in Colorectal Carcinoma (*DCC)*, Endothelin receptor type B (*EDNRB*), $p16^{INK4a}$ and Kinesin chain member 1A (*KIF1A*) in primary OSCCs of Indian origin.

Abstract

We evaluated promoter hypermethylation of a panel of tumor suppressor genes as a means to detect epigenetic alterations in oral squamous cell carcinomas (OSCCs) of Indian-origin and compare with North-American head and neck squamous cell carcinomas (HNSCCs). Quantitative-methylation-specific PCR was used to investigate the promoter methylation status of *DCC*, *EDNRB*, $p16^{INK4a}$ and *KIF1A* in 92 OSCCs, and compared with 48 paired normal tissues and 30 saliva and sera samples from healthy control subjects. Aberrant methylation of at-least one of these genes was detected in 74/92 (80.4%) OSCCs; 72.8% at *EDNRB*, 71.7% at *KIF1A*, 47.8% at $p16^{INK4a}$ and 58.7% at *DCC*; and in 5 of 48 (10.4%) normal oral tissues. None of the saliva and sera samples from controls showed DNA methylation in these four target genes. Thirty-two of 72 node positive cases harbored $p16^{INK4a}$ and *DCC* hypermethylation (p=0.005). Thus, promoter hypermethylation in genes analyzed herein is a common event in Indian OSCCs and may represent promising markers for the molecular staging of OSCC patients. We found higher frequency of $p16^{INK4a}$ methylation (47.8%) in this Indian cohort in comparison with a North-American cohort (37.5%). In conclusion, aberrant methylation of *EDNRB*, *KIF1A*, *DCC* and $p16^{INK4a}$ genes is a common event in Indian OSCCs, suggesting that epigenetic alterations of these genes may be used as potential biomarkers and might play an important role in the pathogenesis of OSCC.

Introduction

Head and neck squamous-cell carcinoma (HNSCC) is the sixth most common cancer in United States and the fourth most prevalent cancer in men worldwide [1]. Rapid advances in treatment modalities and improvements in the early detection of HNSCC have not significantly impacted the overall survival rates of cancer patients (about 50% at 5 years). Development of novel biomarkers offer the potential to transform clinical practice by improving the efficacy of cancer screening and diagnosis based on molecular markers as a complement to routine clinical screening and diagnostic strategies. An epigenetic pathway of transcriptional inactivation for many tumor suppressor genes includes CpG island hypermethylation within promoter regions [2-5]. This pathway has been identified in several human cancers including HNSCC [5-8]. Promoter hypermethylation is a powerful and ubiquitous mechanism of gene silencing which can be detected in tissue samples using quantitative methylation-specific PCR (Q-MSP); this real-time PCR methodology enables an objective, robust, and rapid assessment of promoter methylation status. The ability to quantify the methylation provides the potential for determination of a threshold value of methylation to improve sensitivity and specificity in detection of tumor-specific signal [5, 9].

Some of us (JC, SD) have recently demonstrated the utility of analysis of promoter methylation of a panel of genes in HNSCC. The ongoing discovery efforts to define specific and sensitive promoter methylation based markers for HNSCC and other tumors have resulted in an ever expanding roster of genes with tumor specific hypermethylation [10]. However, there have often been differences in molecular alterations in HNSCC, related to geographic location. In particular, oral squamous cell carcinoma (OSCC) the commonest form of HNSCC in the Indian population have been noted to have a different spectrum of molecular alterations when compared to the North American and European populations [11], perhaps related to differences in risk factors. Defining population based differences in molecular alterations in OSCC would significantly impact understanding of the biology underlying oral carcinogenesis, and indicate a need for population specific approaches to development of novel therapies as well as molecular detection strategies. Even though the gene-promoter methylation data in the oral cavity is meager, measurement of such patterns has shown promise in cancer detection schemes [8]. Because of the sensitivity and specificity of PCR-based assays (especially quantitative methylation-specific PCR) and the potential for use in high-throughput assays, determination of methylation status in oral cancers may have great potential for early detection, monitoring, and treatment.

The objective of this study was to determine the frequency of promoter hypermethylation in a panel of genes (found to be methylated in HNSCC in a North American population by SD and JC), in an Indian cohort. We specifically chose genes that were altered with high specificity, as these genes would be candidates for broad use in molecular detection strategies, if they maintained their specificity across diverse populations. During this study, we were able to show specific differential promoter hypermethylation in Indian OSCC patients compared with normal individuals for four genes, tumor suppressor gene deleted in colorectal carcinoma (*DCC*), Endothelin receptor type B (*EDNRB*), $p16^{INK4a}$ and Kinesin chain member 1A (*KIF1A*). In addition, associations of promoter hypermethylation in these genes with clinicopathological parameters were also assessed.

Material and Methods

Tissue samples: Tissue samples from 92 OSCC patients and 48 normal tissues from a distant site were obtained from patients undergoing surgical treatment in the Institute of Rotary Cancer Hospital (IRCH) at All India Institute of Medical Sciences, using appropriate informed consent obtained after institutional research ethics committee approval. Control saliva rinses and sera from 30 subjects were collected as per the protocol described by Carvalho et al.[5]. Hematoxylin and eosin stained sections were evaluated by the pathologist. Histologically proven cancer and normal tissues were used for DNA extraction as per the method described below.

DNA extraction: DNA from tissues, saliva and sera were extracted by digestion with 50 μg/mL proteinase K (Boehringer, Mannheim, Germany) in the presence of 1% SDS at 48°C overnight, followed by phenol/chloroform extraction and ethanol precipitation.

Quantitative methylation-specific PCR: DNA from primary tumors, adjacent normal tissues from OSCC patients, and saliva and sera from controls were modified with bisulfite and cleaned using EpiTect® bisulfite conversion kit (Cat. No. 59110) purchased from Qiagen Inc, Chatsworth, CA. The bisulfite-modified DNA was used as a template for fluorescence-based real-time PCR, as previously described [6]. In brief, primers and probes were designed to specifically amplify the bisulfite-converted DNA for the *ß-actin* gene (sense, 5'-TGG TGA TGG AGG AGG TTT AGT AAG T-3'; antisense, 5'-AAC CAA TAA AAC CTA CTC CTC CCT TAA-3'; and probe, 6-FAM-5'-ACC ACC ACC CAA CAC ACA ATA ACA AAC ACA-3'-6-TAMRA); *DCC* gene (sense, 5'-TTG TTC GCG ATT TTT GGT TTC-3'; antisense, 5'-AAA AAC CAC TTA CCG ATT ACT TAA AAA-3' and probe, 6-FAM-5'-GCG CTA AAC AAA AAA ACT CCG AAA A-3'-6-

TAMRA) [6]; *p16* gene (sense, 5'-TTA TTA GAG GGT GGG GCG GAT CGC-3'; antisense, 5'-AGT AGT ATG GAG TCG GCG GCG GG-3'; and probe, 6-FAM-5'-GAC CCC GAA CCG CGAC CGT AA-3'-6-TAMRA) [12]; *EDNRB* gene (sense, 5'-GGG AGT TGT AGT TTA GTT AGT TAG GGA GTA G-3'; antisense, 5'-CCC GCG ATT AAA CTC GAA AA-3'; and probe, 6-FAM-5'-TTT TTA TTC GTC GGG AGG AG-3'-6-TAMARA) [13] and *KIF1A* gene (sense, 5'-GCG CGA TAA ATT AGT TGG CGA TT-3'; antisense, 5'-CTC GAC GAC TAC TCT ACG CTAT-3'; and probe, 6-FAM-5'-CCT CCC GAA ACG CTA ATT AAC TAC GCG-3'-6-TAMRA) [13]. The ratios between the values of the gene of interest and the internal reference gene, *ß-actin*, which was obtained by Taqman analysis, were used as a measure for representing the relative level of methylation in a particular sample (gene of interest/reference gene x 100). Fluorogenic PCR reactions were carried out in a reaction volume of 20 µL consisting of 600 nmol/L of each primer; 200 nmol/L probe; 0.75 units platinum Taq polymerase (Invitrogen); 200 µmol/L of each dATP, dCTP, dGTP, and dTTP; 200 nmol/L ROX dye reference (Invitrogen); 16.6 mmol/L ammonium sulfate; 67 mmol/L Trizma (Sigma, St. Louis, MO); 6.7 mmol/L magnesium chloride (2.5 mmol/L for *p16* gene amplification) ; 10 mmol/L mercaptoethanol; and 0.1% DMSO. Three microliters of treated DNA solution were used in each real-time methylation-specific PCR (MSP) reaction. Amplifications were carried out in 384-well plates in a 7900 Sequence Detector System (Perkin-Elmer Applied Biosystems, Norwalk, CT). Thermal cycling was initiated with a first denaturation step at 95°C for 2 minutes, followed by 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. Leukocytes from a healthy individuals were methylated *in vitro* with excess SssI methyltransferase (New England Biolabs) to generate completely methylated DNA, and serial dilutions of this DNA were used for constructing the calibration curves on each plate. Each reaction was carried out in triplicate.

Statistical analysis: Hypermethylation of each gene was treated as a binary variable (methylation versus no methylation) by dichotomizing each gene. Proportions of gene methylation were compared between tumor samples (from cases) and normal tissues (adjacent to tumor tissues) and salivary rinses or sera samples (from controls) using Fisher's exact test. The potential of the confounding effect of the covariates, including age, gender, tobacco and alcohol consumption, was assessed using regression analysis.

Results

OSCC patients and control characteristics: OSCC patients were mainly males (79%) with age ranging from 23 to 72 years (median, 46.5 years) (Table 1). The control population was also mainly males (77% with age ranging from 21 to 71 years; median, 37.5 years). Tobacco consumption was observed in 87% of OSCC patients and 60% control subjects.

Promoter methylation in OSCCs: QMSP was used to analyze the promoter methylation status of four genes in 92 primary OSCCs, 48 matched normal tissues, and sera and saliva from 30 healthy individuals (Table 2). Aberrant methylation was observed in at least one of these genes in 74 of 92 (80.4%) OSCCs. Two genes were methylated in 70 of 92 (76.1%) tumors; three genes were methylated in 55 of 92 (59.8%) tumors. *EDNRB* was methylated in 67 of 92 (72.8%) tumors, *KIF1A* was methylated in 66 of 92 (71.7%) tumors, *p16* was methylated in 44 of 92 (47.8%) tumors and *DCC* was methylated in 54 of 92 (58.7%) tumors.

No methylation was observed in any of the four genes in DNA isolated from sera and saliva of healthy individuals; however three of the histologically proven normal tissues obtained from cancer patients showed methylation in the four genes analyzed in this cohort (Figure 1a-d). Significant association was observed between methylation status of the four genes analyzed in this cohort of OSCC patients (Table 3).

Relationships between promoter methylation and clinico-pathological parameters of OSCC patients: We performed an analysis of clinical and pathologic variables associated with patients with primary OSCC, including age, gender, stage, grade, site, and tobacco and alcohol exposure with methylation status of *DCC*, *EDNRB*, *p16*^{INK4a} and *KIF1A*. No associations with tumor-related factors were noted with methylation status of the genes analyzed except *p16*. *p16* promoter methylation was significantly associated with nodal involvement [p=0.04, OR=3.3, 95% C.I.=1.1-10.2]. Thirty nine of 72 (54.2%) of the node positive OSCC patients showed methylated *p16* promoter. Even when tumor size, differentiation and tobacco consumption were taken into consideration, *p16* methylation was significantly associated with nodal involvement in multivariate analysis [p=0.007, OR=9.1, 95% C.I.=1.8-45.6] (Table 4a). Further, when different combinations of genes were evaluated, OSCCs harboring both *p16* and *DCC* methylation emerged to be more significantly associated with nodal involvement than *p16* alone [p= 0.005, OR=19.2, 95% C.I.=2.4-152.8] (Table 4b). Importantly, 31 of the 56 (55.4%) OSCC patients with tobacco chewing habit were found to have ≥ 3 genes methylated (MI ≥ 0.75, p=0.056, OR=2.4, 95% C.I.=1.0-5.7).

Discussion

Using QMSP-PCR, we observed promoter hypermethylation in at least one of the four genes analyzed in 80.4% of primary OSCCs. Among this panel of genes, *EDNRB* and *KIF1A* were methylated in more than 70% of the OSCC patients. Importantly, *EDNRB*, *DCC*; Microtubule-dependent motor protein: *KIF1A* and cell cycle regulatory gene: $p16^{INK4a}$ have been investigated in head and neck cancer in North American population by our group [6, 13]. In this study we determined the methylation status of these genes in OSCC and adjacent normal tissues of Indian origin. We have recently shown that *DCC* and $p16^{INK4a}$ are epigenetically inactivated by promoter hypermethylation in HNSCC [5, 6]. *DCC* is a putative conditional tumor-suppressor gene located at chromosome 18q21. It encodes a transmembrane protein with structural similarity to neural cell adhesion molecule [14]. DCC promoter region has been found to be hypermethylated in multiple tumor types [6, 15, 16]. p16 is a recognized tumor suppressor which induces G_1 cell cycle arrest by inhibiting the phosphorylation of pRb by the cyclin-dependent kinases CDK4 and CDK6 [17]. EDNRB is a non-selective G protein-coupled receptor which activates phosphatidylinositol-calcium second messenger system and is proposed to negatively regulate the activity of ET-1 which includes ET-1 clearance, inhibition of ET-1 secretion, and activation of signaling transduction pathways that counter-regulate ET-1 [18]. ET_AR mediated mitogenic and additive proliferative effect of ET-1 may be enhanced by methylation inactivation of the *EDNRB* gene and the loss of its unique negative regulatory functions [19]. It has been demonstrated to be hypermethylated in some types of tumors viz., nasopharyngeal, prostate, bladder, hepatocellular, lung, esophageal [20-25]. Kinesin superfamily proteins (KIFs) constitute a large superfamily of microtubule-dependent proteins that transport membranous organelles and macromolecules fundamental for cellular functions along microtubules [26]. KIF1A is an anterograde motor protein that transports membranous organelles along axonal microtubules. Several studies have investigated the effects of *p16*^{INK4a} methylation in primary OSCC [27-29]. Twenty three–87% of primary tumors have methylated *p16*^{INK4a}, making methylation of this gene a very important component of marker panels for tumor screening and detection [30-33]. Our study showed methylated *p16*^{INK4a} in 47.8% OSCCs. *p16*^{INK4a} methylation was observed in 36.4% (32/88) of head and neck cancer patients (oral cavity, oropharynx, larynx, neck) in North American population. The methylation level was 35.1% (13/37) in tumor samples of patients with cancer in the oropharynx region (n=37). The methylation level was 37.5% (9/24) in tumor samples of patients with cancer in the oral cavity (n=24) of the North American population (Demokan S. et al., unpublished results) as compared to 47.8% in the present study in OSCC of Indian population. *DCC* was observed to be methylated in 59% OSCCs. Ogi *et al* [34] showed promoter methylation of *DCC* gene in 16 of 96 primary OSCCs. *DCC* methylation was correlated with bone invasion, and deep invasion of the tongue. Primary oral cancer patients with methylated DCC had a significantly reduced survival than those who did not have DCC methylation [34]. The presence of *p16*^{INK4a} or *DCC* methylation in the primary tumor has been shown to be associated with poor outcome [32, 34, 35]. Our study supports these findings. *p16*^{INK4a} methylation was found to be significantly associated with nodal involvement. Although the presence of *DCC* methylation alone was not a predictor of nodal involvement, combination with *p16*^{INK4a} emerged to be more significantly associated than *p16*^{INK4a} alone. The impact of *p16*^{INK4a} and *DCC* methylation in the primary tumor on nodal involvement was not related to histology or the tumor size. Thirty two of the 34 cases harboring promoter hypermethylation of *DCC* and $p16^{INK4a}$ showed nodal involvement. Thus, aberrant methylation of *EDNRB*, *KIF1A*, *DCC* and $p16^{INK4a}$ genes is highly prevalent in OSCC and may play an important role in the pathogenesis of OSCC.

OSCC in India often has a different etiology, in that these cancers are usually associated with betel quid use and/or tobacco exposure. This study examines a different ethnic cohort from a different geographic region with different exposures than those found in often reported North American cohorts. However, there are remarkable similarities in findings regarding a panel of differentially hypermethylated tumor suppressor gene candidates. These data show that promoter hypermethylation of specific genes in an Indian cohort, including *EDNRB*, *KIF1A*, *DCC* and $p16^{INK4a}$ are fairly comparable to alterations in the North American cohort. This suggests that the epigenetic alterations required to induce OSCC may be fairly consistent despite differences in geography, ethnicity, and exposure patterns. In a practical sense, this also supports the idea that development of prognostic and detection markers in OSCC may be applied across geographic populations and differing carcinogenic exposures with some success.

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References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ. Cancer statistics, 2008. CA Cancer J Clin 2008;58:71-96.

2. Clark SJ, Melki J. DNA methylation and gene silencing in cancer: which is the guilty party? Oncogene 2002;21:5380-7.

3. Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, Baylin SB, Sidransky D. 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. Nat Med 1995;1:686-92.

4. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 2003;349:2042-54.

5. Carvalho AL, Jeronimo C, Kim MM, Henrique R, Zhang Z, Hoque MO, Chang S, Brait M, Nayak CS, Jiang WW, Claybourne Q, Tokumaru Y, et al. Evaluation of promoter hypermethylation detection in body fluids as a screening/diagnosis tool for head and neck squamous cell carcinoma. Clin Cancer Res 2008;14:97-107.

6. Carvalho AL, Chuang A, Jiang WW, Lee J, Begum S, Poeta L, Zhao M, Jeronimo C, Henrique R, Nayak CS, Park HL, Brait MR, et al. Deleted in colorectal cancer is a putative conditional tumor-suppressor gene inactivated by promoter hypermethylation in head and neck squamous cell carcinoma. Cancer Res 2006;66:9401-7.

7. Calmon MF, Colombo J, Carvalho F, Souza FP, Filho JF, Fukuyama EE, Camargo AA, Caballero OL, Tajara EH, Cordeiro JA, Rahal P. Methylation profile of genes CDKN2A (p14 and p16), DAPK1, CDH1, and ADAM23 in head and neck cancer. Cancer Genet Cytogenet 2007;173:31-7.

8. Ha PK, Califano JA. Promoter methylation and inactivation of tumour-suppressor genes in oral squamous-cell carcinoma. Lancet Oncol 2006;7:77-82.

9. Cottrell SE, Laird PW. Sensitive detection of DNA methylation. Ann N Y Acad Sci 2003;983:120-30.

10. Hoque MO, Kim MS, Ostrow KL, Liu J, Wisman GB, Park HL, Poeta ML, Jeronimo C, Henrique R, Lendvai A, Schuuring E, Begum S, et al. Genome-wide

promoter analysis uncovers portions of the cancer methylome. Cancer Res 2008;68:2661-70.

11. Ralhan R, Kaur J. Tobacco associated molecular alterations. In: Pandey M, Nair MK, Sebastian P. Advances in Oncologyed., vol. 2: Jaypee Brothers Med Publishers (P) Ltd, New Delhi, 2002:128-54.

12. Harden SV, Tokumaru Y, Westra WH, Goodman S, Ahrendt SA, Yang SC, Sidransky D. Gene promoter hypermethylation in tumors and lymph nodes of stage I lung cancer patients. Clin Cancer Res 2003;9:1370-5.

13. Demokan S, Chang X, Chuang A, Mydlarz WK, Kaur J, Huang P, Khan Z, Khan T, Ostrow KL, Brait M, Hoque MO, Liegeois NJ, et al. KIF1A and EDNRB are differentially methylated in primary HNSCC and salivary rinses. Int J Cancer 2009:submitted.

14. Fearon ER, Pierceall WE. The deleted in colorectal cancer (DCC) gene: a candidate tumour suppressor gene encoding a cell surface protein with similarity to neural cell adhesion molecules. Cancer Surv 1995;24:3-17.

15. Tan HK, Saulnier P, Auperin A, Lacroix L, Casiraghi O, Janot F, Fouret P, Temam S. Quantitative methylation analyses of resection margins predict local recurrences and disease-specific deaths in patients with head and neck squamous cell carcinomas. Br J Cancer 2008;99:357-63.

16. Park HL, Kim MS, Yamashita K, Westra W, Carvalho AL, Lee J, Jiang WW, Baek JH, Liu J, Osada M, Moon CS, Califano JA, et al. DCC promoter hypermethylation in esophageal squamous cell carcinoma. Int J Cancer 2008;122:2498-502.

17. Tripathi Bhar A, Banerjee S, Chunder N, Roy A, Sengupta A, Roy B, Roychowdhury S, Panda CK. Differential alterations of the genes in the CDKN2A-CCND1-CDK4-RB1 pathway are associated with the development of head and neck squamous cell carcinoma in Indian patients. J Cancer Res Clin Oncol 2003;129:642-50.

18. Smollich M, Wulfing P. The endothelin axis: a novel target for pharmacotherapy of female malignancies. Curr Vasc Pharmacol 2007;5:239-48.

19. Pflug BR, Zheng H, Udan MS, D'Antonio JM, Marshall FF, Brooks JD, Nelson JB. Endothelin-1 promotes cell survival in renal cell carcinoma through the ET(A) receptor. Cancer Lett 2007;246:139-48.

20. Lo KW, Tsang YS, Kwong J, To KF, Teo PM, Huang DP. Promoter hypermethylation of the EDNRB gene in nasopharyngeal carcinoma. Int J Cancer 2002;98:651-5.

21. Zhou L, Feng X, Shan W, Zhou W, Liu W, Wang L, Zhu B, Yi H, Yao K, Ren C. Epigenetic and genetic alterations of the EDNRB gene in nasopharyngeal carcinoma. Oncology 2007;72:357-63.

22. Jeronimo C, Henrique R, Campos PF, Oliveira J, Caballero OL, Lopes C, Sidransky D. Endothelin B receptor gene hypermethylation in prostate adenocarcinoma. J Clin Pathol 2003;56:52-5.

23. Hsu LS, Lee HC, Chau GY, Yin PH, Chi CW, Lui WY. Aberrant methylation of EDNRB and p16 genes in hepatocellular carcinoma (HCC) in Taiwan. Oncol Rep 2006;15:507-11.

24. Chen SC, Lin CY, Chen YH, Fang HY, Cheng CY, Chang CW, Chen RA, Tai HL, Lee CH, Chou MC, Lin TS, Hsu LS. Aberrant promoter methylation of EDNRB in lung cancer in Taiwan. Oncol Rep 2006;15:167-72.

25. Yates DR, Rehman I, Abbod MF, Meuth M, Cross SS, Linkens DA, Hamdy FC, Catto JW. Promoter hypermethylation identifies progression risk in bladder cancer. Clin Cancer Res 2007;13:2046-53.

26. Aprodu I, Soncini M, Redaelli A. Interaction forces and interface properties of KIF1A kinesin-alphabeta tubulin complex assessed by molecular dynamics. J Biomech 2008.

27. O'Regan EM, Toner ME, Finn SP, Fan CY, Ring M, Hagmar B, Timon C, Smyth P, Cahill S, Flavin R, Sheils OM, O'Leary JJ. p16(INK4A) genetic and epigenetic profiles differ in relation to age and site in head and neck squamous cell carcinomas. Hum Pathol 2008;39:452-8.

28. von Zeidler SV, Miracca EC, Nagai MA, Birman EG. Hypermethylation of the p16 gene in normal oral mucosa of smokers. Int J Mol Med 2004;14:807-11.

29. Kresty LA, Mallery SR, Knobloch TJ, Song H, Lloyd M, Casto BC, Weghorst CM. Alterations of p16(INK4a) and p14(ARF) in patients with severe oral epithelial dysplasia. Cancer Res 2002;62:5295-300.

30. Viswanathan M, Tsuchida N, Shanmugam G. Promoter hypermethylation profile of tumor-associated genes p16, p15, hMLH1, MGMT and E-cadherin in oral squamous cell carcinoma. Int J Cancer 2003;105:41-6.

31. Kulkarni V, Saranath D. Concurrent hypermethylation of multiple regulatory genes in chewing tobacco associated oral squamous cell carcinomas and adjacent normal tissues. Oral Oncol 2004;40:145-53.

32. Sailasree R, Abhilash A, Sathyan KM, Nalinakumari KR, Thomas S, Kannan S. Differential roles of p16INK4A and p14ARF genes in prognosis of oral carcinoma. Cancer Epidemiol Biomarkers Prev 2008;17:414-20.

33. Sinha P, Bahadur S, Thakar A, Matta A, Macha M, Ralhan R, Gupta SD. Significance of promoter hypermethylation of p16 gene for margin assessment in carcinoma tongue. Head Neck 2009.

34. Ogi K, Toyota M, Ohe-Toyota M, Tanaka N, Noguchi M, Sonoda T, Kohama G, Tokino T. Aberrant methylation of multiple genes and clinicopathological features in oral squamous cell carcinoma. Clin Cancer Res 2002;8:3164-71.

35. Hamilton SR. Molecular genetic alterations as potential prognostic indicators in colorectal carcinoma. Cancer 1992;69:1589-91.

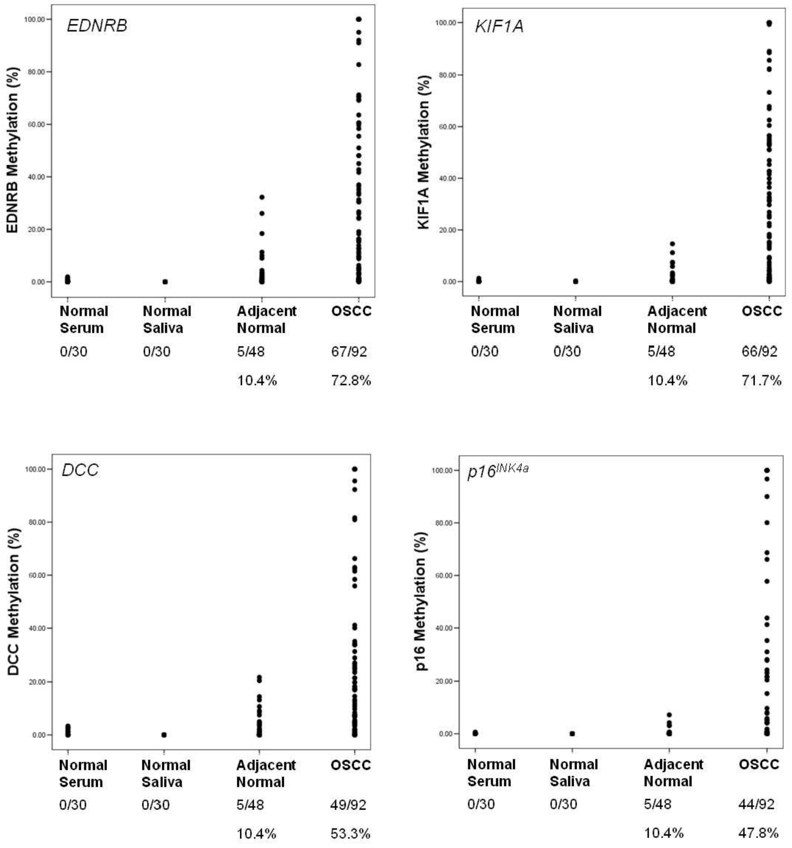

Figure 1: Patterns of promoter hypermethylation observed in tumor DNA and Normal DNA for EDNRB, KIF1A, DCC, and p16INK4a genes
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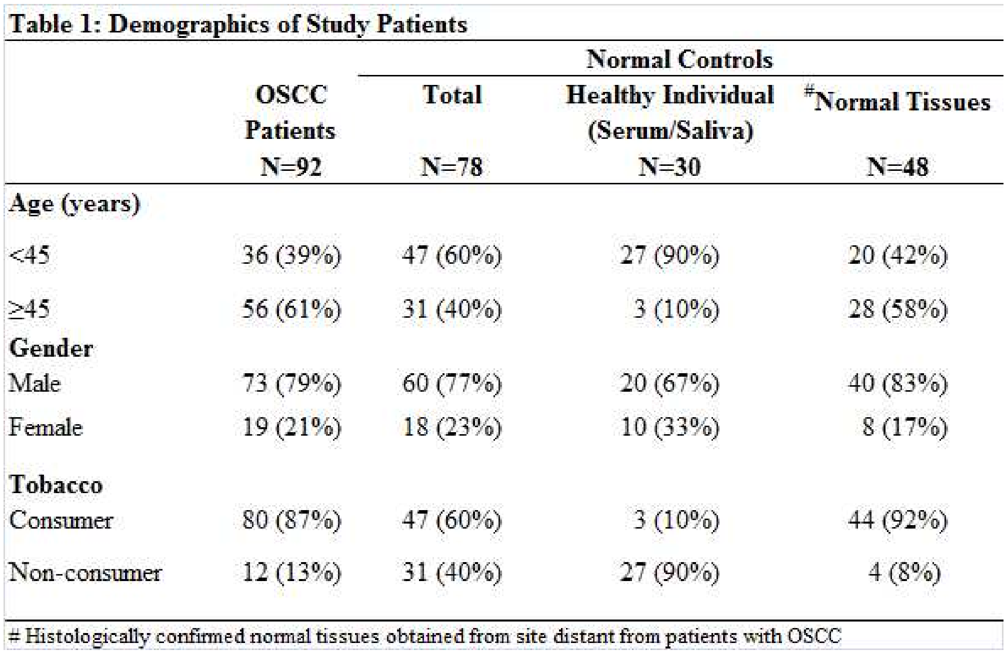

Table 1: Demographics of Study Patients

	OSCC Patients N=92	Normal Controls Total N=78	 Healthy Individual (Serum/Saliva) N=30	 #Normal Tissues N=48
Age (years)				
<45	36 (39%)	47 (60%)	27 (90%)	20 (42%)
≥45	56 (61%)	31 (40%)	3 (10%)	28 (58%)
Gender				
Male	73 (79%)	60 (77%)	20 (67%)	40 (83%)
Female	19 (21%)	18 (23%)	10 (33%)	8 (17%)
Tobacco				
Consumer	80 (87%)	47 (60%)	3 (10%)	44 (92%)
Non-consumer	12 (13%)	31 (40%)	27 (90%)	4 (8%)

Histologically confirmed normal tissues obtained from site distant from patients with OSCC

208x135mm (72 x 72 DPI)

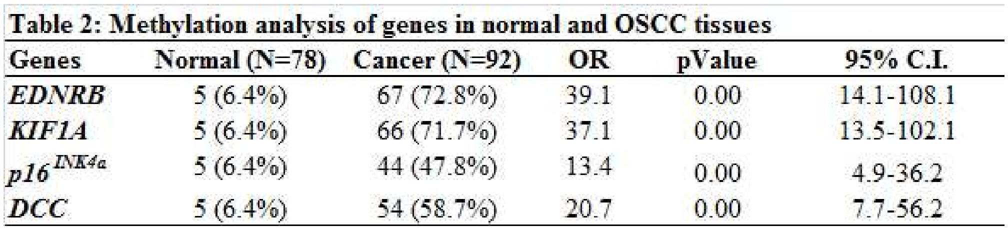

Table 2: Methylation analysis of genes in normal and OSCC tissues

Genes	Normal (N=78)	Cancer (N=92)	OR	pValue	95% C.I.
EDNRB	5 (6.4%)	67 (72.8%)	39.1	0.00	14.1-108.1
KIF1A	5 (6.4%)	66 (71.7%)	37.1	0.00	13.5-102.1
$p16^{INK4a}$	5 (6.4%)	44 (47.8%)	13.4	0.00	4.9-36.2
DCC	5 (6.4%)	54 (58.7%)	20.7	0.00	7.7-56.2

208x46mm (72 x 72 DPI)

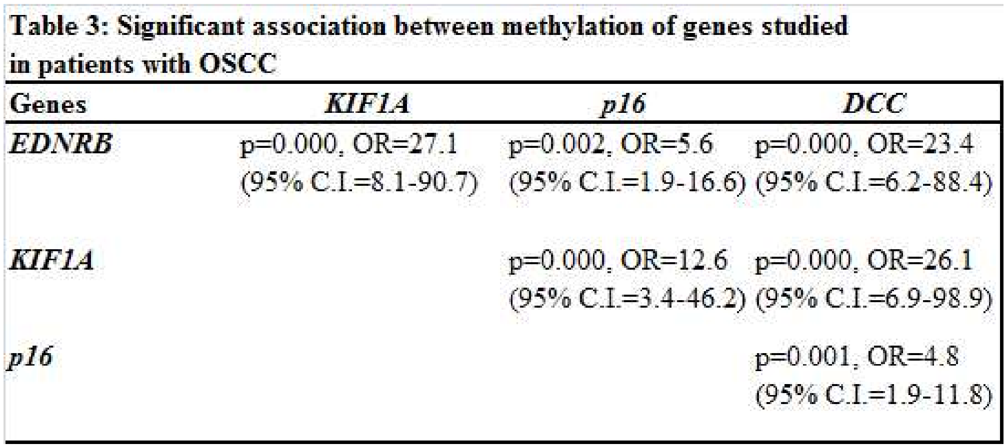

Table 3: Significant association between methylation of genes studied in patients with OSCC

Genes	*KIF1A*	*p16*	*DCC*
EDNRB	p=0.000, OR=27.1 (95% C.I.=8.1-90.7)	p=0.002, OR=5.6 (95% C.I.=1.9-16.6)	p=0.000, OR=23.4 (95% C.I.=6.2-88.4)
KIF1A		p=0.000, OR=12.6 (95% C.I.=3.4-46.2)	p=0.000, OR=26.1 (95% C.I.=6.9-98.9)
p16			p=0.001, OR=4.8 (95% C.I.=1.9-11.8)

193x85mm (72 x 72 DPI)

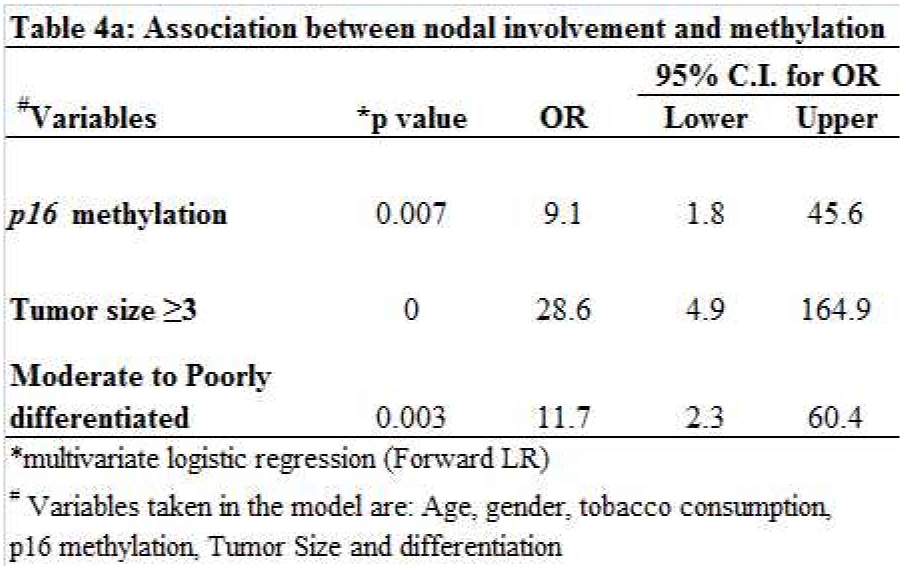

Table 4a: Association between nodal involvement and methylation

#Variables	*p value	OR	95% C.I. for OR	
			Lower	Upper
p16 methylation	0.007	9.1	1.8	45.6
Tumor size ≥3	0	28.6	4.9	164.9
Moderate to Poorly differentiated	0.003	11.7	2.3	60.4

*multivariate logistic regression (Forward LR)

Variables taken in the model are: Age, gender, tobacco consumption, p16 methylation, Tumor Size and differentiation

158x99mm (72 x 72 DPI)

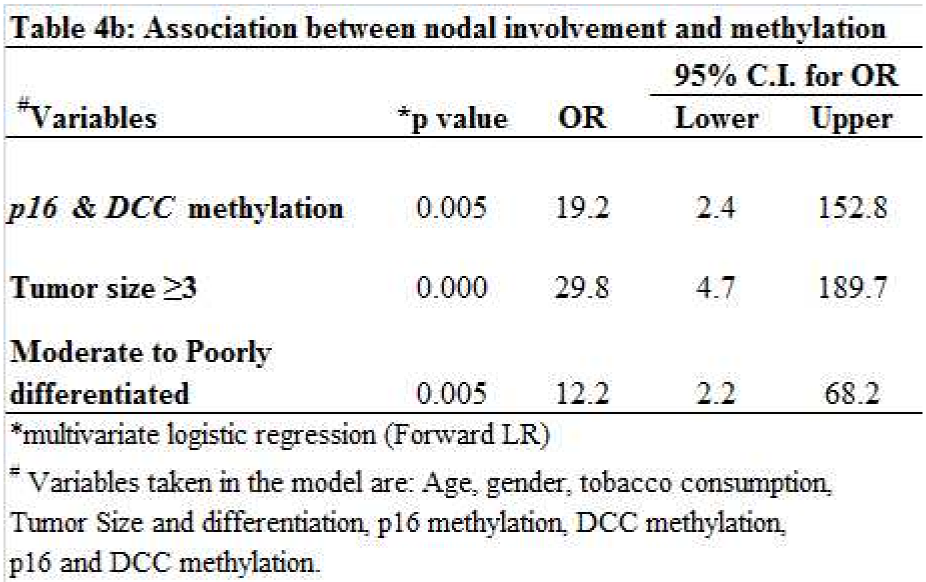

Table 4b: Association between nodal involvement and methylation

			95% C.I. for OR	
[#]Variables	***p value**	**OR**	**Lower**	**Upper**
***p16* & *DCC* methylation**	0.005	19.2	2.4	152.8
Tumor size ≥3	0.000	29.8	4.7	189.7
Moderate to Poorly differentiated	0.005	12.2	2.2	68.2

*multivariate logistic regression (Forward LR)

[#] Variables taken in the model are: Age, gender, tobacco consumption, Tumor Size and differentiation, p16 methylation, DCC methylation, p16 and DCC methylation.

161x101mm (72 x 72 DPI)