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PROMOTER HYPERMETHYLATION PROFILE OF TUMOR-ASSOCIATED GENES *p16, p15, hMLH1, MGMT* AND *E-CADHERIN* IN ORAL SQUAMOUS CELL CARCINOMA

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Aberrant promoter hypermethylation of tumor-associated genes leading to their inactivation is a common event in many cancer types. Using a sensitive restriction-multiplex PCR method, we studied the promoter hypermethylation profile of the *p16, p15, hMLH1, MGMT* and *E-cad* genes in oral squamous cell carcinoma (OSCC) of Indians. We analyzed a total of 51 samples for the *p15* tumor-suppressor gene and 99 samples for each of the remaining genes. Our studies indicate an incidence of promoter hypermethylation of 23% each for *p16* and *p15,* 8% for *hMLH1,* 41% for *MGMT* and 35% for *E-cad.* We observed aberrant hypermethylation of the promoter region of at least 1 of these genes in 74.5% of cases (n = 51) for which all the 5 genes were studied. Abnormal methylation was detected in tumors irrespective of stage and location in the oral cavity, whereas no abnormal methylation was detectable in normal oral squamous tissues obtained from 25 OSCC patients. Detection of aberrant hypermethylation patterns of cancer-associated genes listed above is therefore suitable for diagnosis of OSCC in individuals at high risk for this disease.

Key words: *promoter hypermethylation; oral squamous cell carcinoma;* p16; p15; hMLH1; MGMT; E-cadherin

Oral squamous cell carcinoma (OSCC) is one of the most widely prevalent cancer types in developing countries, including India, and is associated with tobacco and alcohol abuse.[1] Although recent research has given deeper insight into the etiology of the disease, its occurrence as a potentially fatal disease continues unabated. It is therefore essential to identify and develop newer risk markers for diagnosis and therapy of OSCC.

Methylation profiling of promoter regions is essential for understanding the regulation of imprinted genes, X-chromosome inactivation and the role of various tumor-suppressor genes in the genesis of different types of cancer.[2,3] Methylation profiling of tumor-suppressor genes is a potentially powerful diagnostic tool for early detection of various types of cancer.[3–5] Assessment of methylation inactivation is important in assigning functional roles of genes and may have implications in the function of other interacting proteins.[3,6]

Inactivation of several tumor-associated genes, especially tumor-suppressor genes, has been attributed to aberrant hypermethylation of their promoter regions.[2,3,6] We included in our study 5 tumor-suppressor genes: *p16,* a CDK inhibitor involved in regulation of the cell cycle by the cyclin D–Rb pathway, control of which is lost in virtually all tumor types;[7] *p15,* another CDK inhibitor involved in cell-cycle regulation;[8] *hMLH1,* the human homolog of bacterial *MutL,* involved in mismatch repair;[9] *MGMT,* the gene involved in repair of methylated guanosine residues formed due to alkylated carcinogens; and *E-cadherin,* involved in homotypic epithelial cell–cell adhesion.[10] All of these genes are known to be inactivated by methylation in various cancers.[3,11]

Methylation profiling of CpG sites originally involved digestion of sample DNA using a methylation-sensitive restriction enzyme followed by Southern hybridization[7,12] or PCR using primers flanking the restriction site.[13–15] Using these assays, methylation patterns could not be determined from very small amounts of DNA and the occurrence of false-negatives due to degradation or loss of DNA and false-positives due to incomplete digestion could not be excluded.

Methylation-specific PCR (MSP) involves bisulfite modification by deamination of unmethylated cytosines to uracil, followed by PCR amplification using 2 sets of primers, 1 specific for the methylated sequence and 1 for the unmethylated sequence, and, if necessary, direct sequencing using the methylation-specific primers.[16,17] This technique is widely used, and quantification of methylated alleles is possible using fluorescence-labeled primers and light cycler equipment.[18] Given all of the advantages, the assay is difficult to optimize for all loci and expensive methods to purify the DNA after bisulfite modification are needed.

We have developed a multiplex, PCR-based assay for the detection of methylation. In this assay, we employ an appropriate methylation-sensitive restriction enzyme (HpaII) to detect CpG methylation in its recognition site 5′CCGG 3′, which is fairly distributed in CpG islands in the promoter regions of various genes. Using this assay, we have found that the *MGMT* and *E-cadherin* genes are highly methylated in their promoter regions, while the *p16* and *p15* genes are moderately methylated in Indian OSCC patients.

MATERIAL AND METHODS

Sample collection and DNA isolation

Surgically resected OSCC samples were collected from Government Rajaji Hospital, Madurai; transferred to cold PBS; and transported on ice to the laboratory. DNA was isolated immediately by proteinase-K digestion, followed by organic extraction.

Restriction digestion of genomic DNA

Genomic DNA (3 μg) was digested initially with 5 U of HpaII restriction enzyme (Amersham, Aylesbury, UK) in 1 × supplied buffer L for 2 hr at 37°C, and the reaction was refreshed with 10 U of HpaII incubated overnight. Phenol-chloroform extraction was done immediately after digestion. Restriction digestion of genomic DNA was ascertained by PCR using primers for the human protein phosphatase gene exon 1 region, which were designed flanking the HpaII restriction site. Absence of amplified product compared to control indicated complete digestion.

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Primers

Promoter regions of the tumor-associated genes used in our study were obtained from the NCBI database, and the primers were designed flanking HpaII sites (5′CCGG3′) within the region reported to be methylated (usually 500 bp of the transcription start site), as shown in Table I.

TABLE I – SEQUENCE OF PRIMERS AND AMPLICONS

No.	Gene	Primer	Sequence	M.Wt SA/NA (bp)	No. of *HpaII* sites included	Primer Conc. (μM)	5% DMSO requirement
1	*p16*	*p16 S*	5′ GAAGAAAGAGGAGGGGCTG 3′	SA-340 NA-170	2	0.2	Yes
		p16 A	5′ GGTCGGGTAGAGGAGGTGC 3′			0.4	
		p16 N	5′ GCGCTACCTGATTCCAATTC 3′			0.2	
2	*p15*	*p15 S*	5′ CGAGGCGGGGCAGTGAG 3′	SA-234 NA-126	2	0.4	No
		p15 A	5′ TCAAGAACCAGCGGGCG 3′			0.2	
		p15 N	5′ CTAGGAGACCTGGGCTCAGC 3′			0.2	
3	*hMLH1*	*hMLH1* S	5′ GGAGAGGAGGAGCCTGAGAAG 3′	SA-392 NA-255	2	0.2	Yes
		hMLH1 A	5′ AGATGCTCAACGGAAGTGCCT 3′			0.4	
		hMLH1 N	5′ GAACCAATAGGAAGAGCGGAC 3′			0.2	
4	*E-cadherin*	*E-cad* S	5′ GGCAATACAGGGAGACACAGC 3′	SA-360 NA-155	1	0.2	No
		E-cad A	5′ GCACGGTTCTGATTCCACTG 3′			0.2	
		E-cad N	5′ GGCTCAAGCGGTCCTCTGG 3′			0.4	
5	*MGMT*	*MGMT* S	5′ TCTTCCTGTCTCAGCCTTCC 3′	SA-418 NA-253	1	0.2	No
		MGMT A	5′ AATTACTGTATCCCGATTCTTCTC 3′			0.4	
		MGMT N	5′ GCATAGGTGCTGAGTTGAATC 3′			0.2	

SA, amplicon when sense and antisense primers were used; NA, amplicon when nested and antisense primers were used.

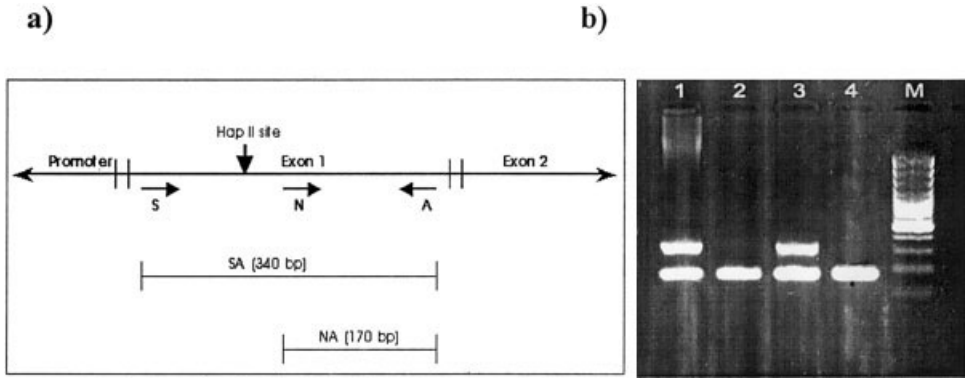

FIGURE 1 – Seminested, multiplex PCR for assessment of methylation. *(a)* Schematic representation showing the strategy for detection of methylation at the 5′CCGG3′ HpaII restriction site in the promoter region of the *p16* tumor-suppressor gene. *(b)* Agarose gel electrophoretic pattern of amplified products using sense, antisense and nested primers in a multiplex reaction. Lane 1, *in vitro* methylated *p16* promoter plasmid construct used as template; lane 2 unmethylated promoter plasmid construct; lane 3, *in vitro* methylated human placental DNA; lane 4, human placental DNA; lane M, 100 bp DNA ladder.

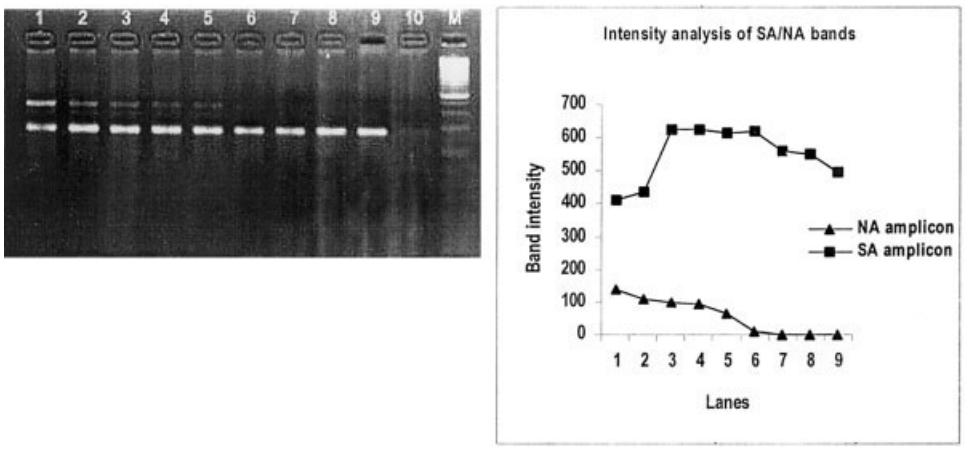

FIGURE 2 – *(a)* Detection of methylation in a fraction of target sequences by the assay. PCR products electrophoresed in 2% agarose gel. Promoter plasmid construct pSA and pNA were spiked in different ratios into 50 ng/μl salmon sperm DNA, and PCR was performed. Lane 1, pSA:pNA 1:1; lane 2, pSA:pNA 1:2: lane 3, pSA:pNA 1:3; lane 4, pSA:pNA 1:4; lane 5, pSA:pNA 1:5; lane 6, pSA:pNA 1:6; lane 7, pSA:pNA 1:7; lane 8, pSA:pNA 1:8; lane 9, pSA:pNA 0:1; lane 10, 50 ng salmon sperm DNA (carrier control); lane M, 100 bp DNA ladder. *(b)* Intensity plot of the semiquantitative assay. Lanewise intensity plot of Figure 3 analyzed on an Alpha Innotech (San Leandro, CA) Image quant gel-documentation system. SA, amplicon when sense and antisense primers were used; NA, amplicon when nested and antisense primers were used.

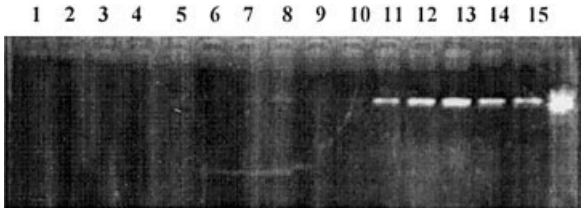

FIGURE 3 – Verification of complete HpaII digestion. Representative figure showing PCR products obtained using primers for human protein phosphatase flanking a single HpaII site. Lanes 1–10, HpaII-digested DNA template; lanes 11–15, undigested controls.

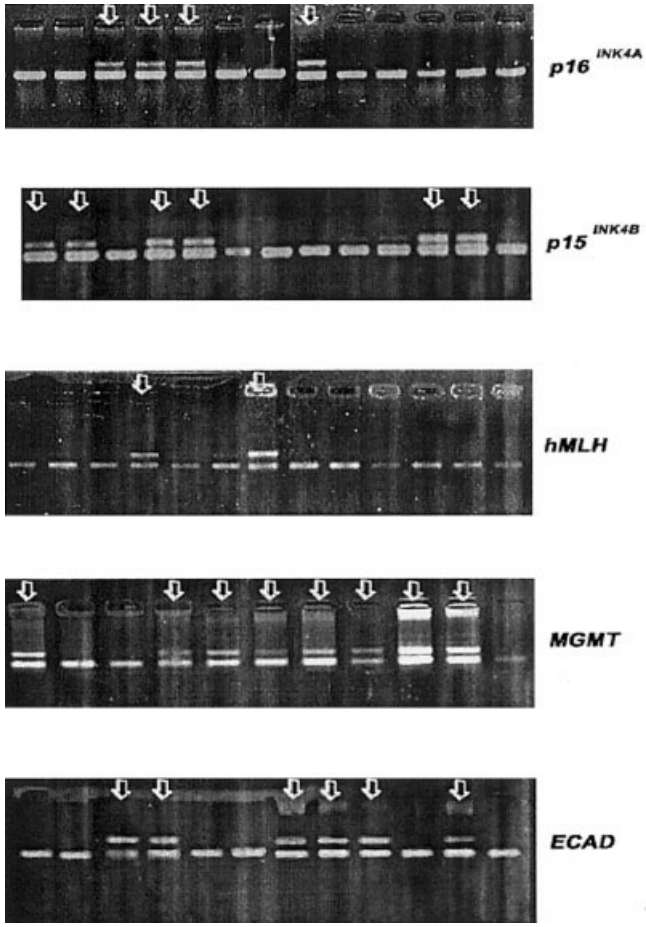

FIGURE 4 – Agarose gel electrophoretic pattern of hypermethylation. Representative samples of promoter hypermethylation for the 5 genes are shown. Hypermethylation-positive cases are marked with arrows.

PCR conditions

Seminested multiplex PCRs were carried out, unless otherwise mentioned, in a 10 μl reaction using 50 ng of template DNA, 1.5 mM $MgCl_2$, 1 × PCR buffer, 200 μM dNTPs, 5.0% DMSO, 0.2 μM each of sense nested and 0.4 μM of antisense primers and 0.25 U of Promega (Madison, WI) Taq DNA polymerase.

Consensus cycling parameters

The consensus thermal cycling conditions optimized to amplify all amplicons of the 5 candidate genes under study simultaneously on a Perkin Elmer (Oak Brook, IL) Gene-Amp 2400 thermocycler were as follows: initial denaturation at 95°C for 3 min followed by touch-down cycling at 95°C for 30 sec, 62°C for 1 min (incubation temperature was reduced by 0.5°C each cycle up to 54°C and

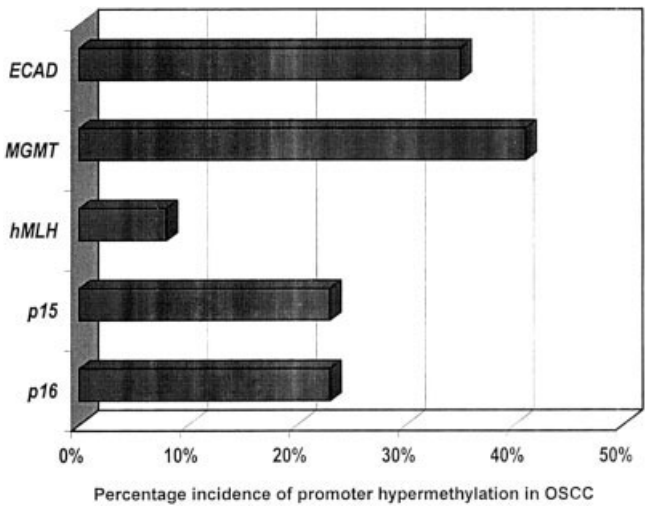

FIGURE 5 – Incidence of promoter hypermethylation. The percentage incidence for the genes *p16, hMLH1, MGMT* and *E-cad* in 99 OSCC samples and *p15* in 51 samples is shown.

incubation time by 1.0 sec each cycle) and 72°C for 30 sec. This was followed by 15 additional cycles at 95°C for 30 sec, 54°C for 45 sec and 72°C for 30 sec.

Intensity plots

Human placental DNA in different concentrations was used as template in multiplex PCR for all genes studied, and the intensity of the low m.w. band *vs.* the high m.w. band was plotted. This was used as a standard to verify the absence/presence of methylation in the HpaII-treated genomic DNA samples.

RESULTS

To assess the methylation profile in cancer-related genes, we designed primers, a pair flanking at least 1 HpaII (5′CCGG3′) site and a seminested primer in the promoter region (DNA sequence up to 500 bp upstream of the transcription start site; the CpGs of these promoters are known to be hypermethylated in human cancers), of the 5 genes used. The strategy involves digestion of the total genomic DNA with restriction enzyme HpaII, followed by seminested, multiplex PCR using primers designed for the promoter region, the outer primers (sense and antisense) amplifying a product flanking the restriction site for the enzyme while the product generated by the seminested and the antisense primers is not affected by the restriction digestion (Fig. 1). Appearance of 2 bands using enzyme-treated genomic DNA as template would indicate methylation of the CpG site(s), while a single band amplified by the nested primer would indicate unmethylated DNA. A consensus PCR condition was developed, which could be used to detect the methylation status of the promoter regions of several genes. A standard plot comparing the intensity of the 2 bands (one generated by the sense and antisense primers and the other by nested and antisense primer) using serial concentrations of genomic DNA template was used to eliminate false-positives and -negatives. For confirmation of the sensitivity of our strategy, human placental DNA and a plasmid vector containing a *p16* promoter insert were methylated *in vitro* using Sss1 methylase and digested using HpaII as described and the precipitated DNA was used as template in the multiplex PCR using the primer set for *p16.* We detected methylated samples as appearance of both full-length and nested products (Fig. 1*b*). Further, using a standard plot of the intensities of the 2 amplicons, we also determined that the assay could detect methylation in 20% of alleles using different dilutions of the cloned *p16* promoter (Fig. 2). Subsequently, the *in vitro* methylated human placental DNA was used as a template to test

TABLE II – CONSOLIDATED DATA SHOWING THE PROMOTER HYPERMETHYLATION PATTERN OF 5 GENES IN OSCC AND CORRELATION WITH OTHER GENE ALTERATIONS STUDIED IN THE LAB

Number	Sample	Epigenetic alterations Promoter hypermethylation analysis					Genetic alterations Mutation and deletion analysis				
		p16	*p15*	*hMLH*	*MGMT*	*E-Cad*	*p53*[23,24]	*ras*[21]	*p16*[26]	*p14*[arf25]	*Fhit*[30]
1	O1				■						
2	O7										
3	O13									del	
4	O21				■						
5	O22	■			■		A-G			del	
6	O23									del	
7	O25	■			■		G-A				
8	O28								G-C		
9	O29										
10	O30				■						
11	O35				■	■					
12	O37										
13	O41				■						
14	O43				■						
15	O44	■									
16	O57					■	C-T				
17	O58										
18	O59		■								A-G
19	O65										
20	O129		■		■	■					
21	O127										
22	O126				■						
23	O124				■	■					
24	O92				■	■					
25	v1										
26	v2				■						
27	v3				■						
28	v4				■						
29	v5										
30	v6	■				■					
31	v7										
32	v8					■					
33	v10										
34	v11		■			■					
35	v12										
36	v13		■								
37	v14				■						
38	v15		■			■					
39	v16	■									
40	v17	■	■		■	■					
41	v18		■		■	■					
42	v19		■	■	■						
43	v20			■	■	■					
44	v21				■	■					
45	v22				■						
46	v23		■	■		■					
47	v24	■	■								
48	v25				■						
49	v26	■	■			■					
50	v27	■		■							
51	v28	■	■	■							
52	1			■	■						
53	2						T-C				
54	3					■	T-C				
55	4										
56	5										
57	6										
58	7					■	T-C				
59	8					■					
60	9					■					
61	10										
62	11					■					
63	12							G-A			
64	13	■									
65	14	■									
66	15	■		■	■			G-T			
67	16					■					
68	17					■		G-T			
69	18	■		■	■						
70	19										
71	20										
72	21										
73	22					■					

TABLE II – CONSOLIDATED DATA SHOWING THE PROMOTER HYPERMETHYLATION PATTERN OF 5 GENES IN OSCC AND CORRELATION WITH OTHER GENE ALTERATIONS STUDIED IN THE LAB (CONTINUED)

Number	Sample	Epigenetic alterations Promoter hypermethylation analysis					Genetic alterations Mutation and deletion analysis				
		p16	*p15*	*hMLH*	*MGMT*	*E-Cad*	*p53*[23,24]	*ras*[21]	*p16*[26]	*p14*[arf][25]	*Fhit*[30]
74	23										
75	24										
76	25										
77	26	■									
78	27										
79	28				■						
80	29								C-T		
81	30										
82	31	■			■						
83	32										
84	T1				■						
85	T2							G-T	C-T		
86	T3										
87	T4										
88	T5										
89	T6										
90	T7				■						
91	T8	■				■					
92	T9										
93	T10				■			G-A			
94	T11	■									
95	T12				■						
96	T13						4bp del				
97	T14				■		T-G				
98	T15	■									
99	T16					■					

Gray boxes, analyzed; white boxes, not analyzed; black boxes, methylated. Superscript numbers in column heads are references cited.

for the other genes. Ninety-nine surgically resected OSCC cases were studied to assess promoter hypermethylation in 4 genes, *p16, MGMT, hMLH1* and *E-cadherin;* and 51 samples of these were checked for methylation of the *p15* promoter region.

The extent of digestion of the DNA samples by HpaII was assessed using sense and antisense primers designed flanking a single HpaII site in the exon 1 region of the human protein phosphatase gene. Complete digestion was indicated by absence of the amplicon compared to undigested control DNA. The same HpaII-treated DNA samples verified by the above process were used to study for promoter hypermethylation in all of the genes studied (Fig. 3).

The percentage incidence of promoter hypermethylation was 23% for both *p15* and *p16.* In the case of *hMLH1,* promoter hypermethylation was detected, albeit in a lower number (8%) of OSCC cases. The promoter region of *E-cadherin* was hypermethylated in 35% of individuals, and aberrant hypermethylation incidence was the highest in 41% of cases for the *MGMT* promoter (Figs. 4,5). For the 51 samples in which methylation status of all 5 genes was studied, 74.5% of cases showed methylation for at least 1 of the genes studied, 40% for 2 of the genes and 16% for 3 of the genes; only 1 sample showed methylation in 4 genes. For the 99 samples in which methylation status of 4 genes were studied, 72% showed aberrant methylation for at least 1 of the genes, 29% for 2 of the genes, 7% for 3 of the genes and 0 for all 4 genes (Table II).

Twenty-five normal samples of squamous tissue of the oral cavity were also included, which did not show any aberrant hypermethylation in the promoter region for the genes studied.

No significant correlation was observed in the hypermethylation pattern of 2 different genes in OSCC; *i.e.,* for the genes used here, a methylation event in the promoter region of 1 gene was not significantly associated with promoter hypermethylation of any other gene.

DISCUSSION

Methylation is a major epigenetic modification in humans, and changes in methylation patterns play an important role in tumorigenesis.[2,6] Regions that are frequent targets for hypermethylation events are CpG islands. CpG islands are GpC- and CpG-rich regions of approximately 1 kb that are usually associated with the promoter or 5′ end of genes.[6] Abnormal methylation of CpG islands can efficiently repress transcription of the associated gene in a manner akin to mutations and deletions and act as one of the "hits" in Knudson's 2-hit hypothesis for tumor generation.[6] Tumor cells exhibit global hypomethylation of the genome accompanied by region-specific hypermethylation events.[6]

In our study, we analyzed only specific CpGs that are generally observed to be aberrantly methylated in human cancers, and this may limit our methylation profile data since methylation patterns of CpGs have been observed to be heterogeneous in their occurrence; *i.e.,* in a particular cancer type, CpG sites methylated in one patient need not be the same as in another patient.[19] A true methylation profile could be generated by bisulfite sequencing, but this would be a cumbersome technique for studying a panel of genes in a large number of samples.

In human OSCC, a profile indicating the extent of methylation of tumor-associated genes remains largely unknown. Our studies indicate that the repair genes *MGMT* and *E-cadherin* are the most preferentially targeted genes for methylation in OSCC of Indians (Table II). Previous studies have indicated a positive correlation between tobacco carcinogens and *MGMT* hypermethylation.[1] Inactivation of *MGMT* leads to transition mutations indicated by accumulation of high levels of mutations of this type in oncogenes and tumor-suppressor genes.[20] Point mutations in the *ras* gene corresponded with promoter hypermethylation of the *MGMT* gene in 60% (3/5) of cases out of 48 samples in which the *ras* mutation status was studied,[21] and for the 67 samples in which mutation status of the *p53* was known,[22,23] the correlation between the transition mutations in *p53* and promoter hypermethylation of *MGMT* gene was 3 of 7 (43%) (Table II).

The *INK4A* locus is altered in a majority of cancer types, including OSCCs;[24,25] specifically, the *p16* tumor-suppressor gene is altered in virtually all tumors. The levels of hypermethylation observed in OSCC are consistent with those observed by other workers in head-and-neck cancer.[20] Previous studies from our laboratory indicated that mutations were infrequent in this gene in

OSCC.[26] High incidence of hypermethylation in the promoter region of this gene is indicative of alternate mechanisms of inactivation. Our observations also indicate that the 2 inactivating events, mutations and promoter hypermethylation, in the *p16* gene did not occur simultaneously in the cases analyzed (Table II).

We have observed a very low incidence of promoter hypermethylation of the *hMLH1* gene, which is involved in mismatch repair function. Loss of expression of this gene leads to genomic instability, manifested in the form of microsatellite instabilities. Low levels of methylation inactivation of *hMLH1* indicated in our study are consistent with the observation of very low incidence of microsatellite instability in OSCC.[27–29]

The main conclusion of our study is that even though the promoter regions of multiple genes could be methylated in an OSCC case, there is no significant correlation in occurrence of promoter hypermethylation between any 2 of the genes studied here. This indicates that promoter hypermethylation might be a random, rather than a targeted, event. The question that remains unanswered is how only specific subsets of genes are hypermethylated in particular cancer types.

The technique devised by us is simple and straightforward; and though problems due to incomplete digestion are likely to arise, use of reliable genomic DNA purification techniques, standardization of PCR sensitivity by reducing end cycles and use of a standard plot curtail false-positives. In our study, the occurrence of false-positives was ruled out as verified by PCR using primers flanking a single HpaII site in the human protein phosphatase gene and further indicated by the fact that none of the samples was positive for all of the genes tested using the same restriction-digested DNA as template.

The studies described here using 5 genes indicate that aberrant promoter hypermethylation occurred in at least 1 of the genes in 74.5% of cases. Our studies suggest that epigenetic mechanisms could play a major role and in some cases may even supersede genetic events in the inactivation of certain tumor-suppressor and tumor-associated genes in the genesis of OSCC.

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