Evaluation of p16 hypermethylation in oral submucous fibrosis: A quantitative and comparative analysis in buccal cells and saliva using real-time methylation-specific polymerase chain reaction

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Abstract

Aims: The aim of this study was to quantitatively investigate the hypermethylation of p16 gene in buccal cells and saliva of oral submucous fibrosis (OSMF) patients using real-time quantitative methylation-specific polymerase chain reaction (PCR) and to compare the values of two methods. **Subjects and Methods:** A total of 120 samples were taken from 60 subjects selected for this study, of which 30 were controls and 30 patients were clinically and histopathologically diagnosed with OSMF. In both groups, two sets of samples were collected, one directly from the buccal cells through cytobrush technique and the other through salivary rinse. We analyzed the samples for the presence of p16 hypermethylation using quantitative real-time PCR. **Results:** In OSMF, the hypermethylation status of p16 in buccal cells was very high (93.3%) and in salivary samples, it was partially methylated (50%). However, no hypermethylation was found in controls suggesting that significant quantity of p16 hypermethylation was present in buccal cells and saliva in OSMF. **Conclusions:** This study indicates that buccal cell sampling may be a better method for evaluation than the salivary samples. It signifies that hypermethylation of p16 is an important factor to be considered in epigenetic alterations of normal cells to oral precancer, i.e. OSMF.

Key words: CpG islands, epigenetic, hypermethylation, oral submucous fibrosis, p16

Introduction

The fifth most common cancer in the world is oral cancer.[1] Approximately, 75,000 ± 80,000 new cases of oral cancer have been documented in India. In all nonrural cancer registries, oral cancer ranks highest among all types of cancers in the world.[2] Oral cancer and oral mucosal diseases such as oral submucous fibrosis (OSMF) and leukoplakia have a close association with the habit of smoking, tobacco, and betel nut chewing.[2] A chronic, progressive, disabling oral mucosal disease with a potential for malignant transformation is OSMF. It is seen predominantly in the South Asians, more prevalent in Indians. In India alone, the statistics for OSMF is about 5 million people (0.5%).[3]

Oral squamous cell carcinoma associated with betel quid causes specific genetic mutations, in the p53 gene. A part from gene mutation, epigenetic alterations such as DNA hypermethylation, histone acetylation, and phosphorylation are often observed in the tumor.[4] DNA methylation takes place in the mammalian DNA molecule predominantly at cytosine bases that are located 5´ to a guanosine. It is basically a covalent biochemical modification.[5]

CpG islands mostly appear near the promoter regions, and it extends to the first exon of specific genes. Hypermethylation and unmethylation state of CpG islands are located in and around the promoter region, plays an important role in regulating gene expression. In normal cells, they are unmethylated and in human cancer they seem to be hypermethylated.[6] In fact, gene silencing occurs commonly due to promoter hypermethylation than genetic mutation. This event occurs earlier, preceding changes in protein expression level in oral carcinogenesis. This pathway makes promoter hypermethylation a very attractive diagnostic marker for the early detection of oral cancer.[7]

p16 is a tumor suppressor gene located at chromosome 9p21. In human cancers, one of the most frequently altered genomic regions is a 9p21 chromosomal band. This region contains cluster of three genes, $p14^{ARF}$, $p15^{INK4b}$, and $p16^{INK4a}$ within a short distance of 50 kb, all of which have putative tumor suppressor roles. Moreover, CpG islands are highly abundant in the promoter regions of all three genes, and they are more susceptible to hypermethylation.[8] In many human cancers including oral cancers, loss of p16 is frequently observed.[9,10] 83% of oral cancer and 60% of the premalignant lesion shows the loss of p16 expression, suggesting that p16 alteration is an early event in oral cancer.[11]

Only very few studies are available on hypermethylation of p16 in oral precancer even though hypermethylation of tumor suppressor genes in malignant tumors including oral cancer has been documented. The detection of p16 hypermethylation in precancer can predict the risk of malignant transformation[12] and may also be a used as a prognostic marker. Hence, this study was aimed to quantitatively investigate the promoter hypermethylation of p16 gene in buccal cells and saliva of OSMF patients using real-time quantitative methylation-specific polymerase chain reaction (PCR) and to compare the values of two methods.

Aim

To quantitatively evaluate p16 hypermethylation in buccal cells and saliva of OSMF patients and to compare the values in these two samples.

Objectives

- To quantitatively determine the presence of p16 hypermethylation in buccal cells and salivary samples of OSMF patients
- To quantitatively determine the presence of p16 hypermethylation in buccal cells and salivary samples of normal healthy individuals
- To compare p16 hypermethylation in buccal cells and salivary samples of OSMF patients.

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Subjects and Methods

Selection of patients

The study protocol was approved by Ethics Committee for student proposal, Sri Ramachandra University. A total of 120 samples were taken from 60 subjects selected for the study, of which 30 were controls (Group A) and 30 were patients with OSMF (Group B). In both groups two sets of samples were collected, one directly from the buccal cells through cytobrush technique and the other through salivary rinse. All 60 patients were reported to the department of oral medicine and radiology. Based on the clinical and histopathological evaluation these patients were confirmed as OSMF. The patient age group for the OSMF ranged from 21 to 67 years with mean age of 44. Out of 30 OSMF patients two of them were female, 28 of them were male. Samples from 30 study group (Group B) were taken from those who had not undergone any form of therapy for the presenting illness. Samples from 30 controls (Group A) who participated in the study were taken from the healthy volunteers of matched age and gender, who were free of oral and medical illness, without any habits of smoking, alcohol, chewing betel nut, and tobacco.[13] The age group for controls (Group A) ranged from 21 to 60 years with mean age of 40 years. The patients and controls were explained about the study, and written consent was taken.

Sampling of exfoliative cytology

Buccal samples collection was done with the help of cytobrush from 30 control group and 30 OSMF patients. All the participants were instructed to rinse the mouth with tap water for 10 s before collection. The buccal mucosa was scraped by simple counter pressure by twirling the brush while moving it downward and the counter pressure was applied with fingers against the external cheek for 30 s, and the brush was stored in a 15 ml centrifuge tube directly without additional processing in −80°C freezer. The Same method was followed for the control group.[14]

Sampling of saliva

Twenty milliliters of salivary rinses were collected by rinsing or gargling, for 60 s with 20 ml of sterile sodium chloride solution at 0.9%, in a 30 ml sterile centrifuge tube from 30 control group, and 30 OSMF patients. The salivary samples collected were transferred to the laboratory and was stored at −80°C.[14]

The samples were subjected to analyses for the presence of p16 hypermethylation using quantitative real-time PCR.

DNA extraction

Genomic DNA from samples of exfoliative cytology and salivary rinse was extracted with QIAamp DNA mini kit with 20 µl of QIAGEN Protease stock solution. DNA from salivary samples and exfoliative cytology of OSMF patients and from controls were modified with bisulfite and cleaned using EpiTect bisulfite conversion kit (Cat. No: 59104) purchased from QIAGEN. Briefly, 2 mg of genomic DNA was denatured in 0.2 mol/L of NaOH for 20 min at 50°C.

Real-time quantitative methylation-specific polymerase chain reaction

The modified DNA was used as a template for fluorescence-based real-time PCR. The quantitative real-time PCR was carried out on a fast real-time PCR 7900HT system. The primers were used to detect methylated p16 gene. ß-actin gene was used as a reference control. The ratio of methylated p16 promoter DNA to ß-actin DNA represented the relative p16 methylation level,[15] all reactions were performed in triplicate in 20 µl of total volume of PCR products. The component of PCR product is given in Table 1.

All reactions were performed with methylated and unmethylated primers and ß-actin endogenous control (positive control) and the nontemplate control (negative control) contained no template DNA.

During the annealing and the extension step of each cycle of real-time PCR, the Ct value of the amount of product amplified by the fluorescence of SYBR Green dye was plotted on an amplification curve. The cycle threshold value obtained was calculated for each amplification in each experimental sample by use of Applied Biosystems 7900HT fast real time pcr system soft ware SDS2.1.[15] The results derived for evaluation of the presence of p16 hypermethylation by real-time PCR.

The comparative Ct method (also called ΔCt) is calculated by formula:

$\Delta Ct = avgCt_{GOI} - avgCt_{ref}$[16]

GOI - gene of interest, ref - the reference gene.

Subsequent to the derived Ct values, the results of p16 hypermethylation was evaluated.

Statistical analysis

Since Group A (controls) showed no methylation (0%), statistically odds ratio cannot be calculated to give a significant *P* value.

Results

The sample collected were analyzed to study the methylation status of p16 gene by quantitative methylation specific PCR and the values are tabulated in Tables 2-5, respectively.

Table 2 shows the methylation status of p16 in buccal cells of Group A by comparing the target gene p16 (Col IV and VIII) with reference gene β-actin (Col III and VII) in unmethylated and methylated primers respectively. Delta Ct values, the difference in threshold cycles for the target gene and reference gene were calculated. The average Delta Ct value of unmethylated p16 gene was 2.332 and methylated 8.388. No methylation was detected in all 30 samples, and the results suggest that Group A (controls) is unmethylated (Col X).

Table 3 shows the methylation status of p16 in buccal cells of Group B by comparing the target gene p16 (Col IV and VIII) with reference gene β-actin (Col III and VII) in the unmethylated and methylated primers respectively. Of 30 samples tested 22 showed methylation, 2 showed

Table 1: Component of PCR products

Serial number	Contents	Volume (µl)
1	SYBR Green mix (×2)	10
2	Forward primer	1
3	Reverse primer	1
4	Template (mid brain cDNA)	1
5	Sterile water	7
Total		20

PCR=Polymerase chain reaction

Table 2: Methylation status of p16 in the buccal cells from exfoliative cytology samples of Group A (controls)

Serial number (Col I)	Unmethylated primer				Methylated primer				Result (Col X)
	Sample name (Col II)	β-actin (Ct) (Col III)	Unmethylated (Ct) (Col IV)	Delta (Ct) (Col V)	Sample name (Col VI)	β-actin (Ct) (Col VII)	Methylated (Ct) (Col VIII)	Delta (Ct) (Col IX)	
1	CE16	21.675	23.664	1.988	CE16	21.675	29.852	8.177	U
2	CE17	21.544	24.235	2.691	CE17	21.544	27.431	5.888	U
3	CE18	20.342	22.970	2.628	CE18	20.342	26.879	6.537	U
4	CE19	21.276	23.887	2.611	CE19	21.276	30.113	8.837	U
5	CE20	20.236	23.112	2.876	CE20	20.236	30.564	10.328	U
6	CE21	20.621	24.324	3.703	CE21	20.621	29.924	9.303	U
7	CE22	20.165	23.445	3.280	CE22	20.165	26.562	6.397	U
8	CE23	20.267	24.998	4.731	CE23	20.267	29.715	9.448	U
9	CE24	20.889	22.225	1.336	CE24	20.889	26.867	5.978	U
10	CE25	22.215	24.342	2.127	CE25	22.215	31.450	9.235	U
11	CE26	21.897	24.665	2.768	CE26	21.897	32.450	10.553	U
12	CE27	21.124	23.667	2.543	CE27	21.124	29.980	8.856	U
13	CE28	20.576	23.888	3.312	CE28	20.576	29.780	9.204	U
14	CE29	22.779	22.892	0.113	CE29	22.779	32.140	9.362	U
15	CE30	21.221	23.124	1.903	CE30	21.221	31.760	10.539	U
16	CE1	22.675	22.879	0.204	CE1	22.675	28.120	5.445	U
17	CE2	22.897	23.908	1.011	CE2	22.897	27.119	4.222	U
18	CE3	22.445	25.233	2.788	CE3	22.445	34.670	12.225	U
19	CE4	21.987	24.556	2.569	CE4	21.987	26.697	4.710	U
20	CE5	23.675	24.453	0.778	CE5	23.675	32.780	9.105	U
21	CE6	20.776	23.988	3.212	CE6	20.776	26.980	6.204	U
22	CE7	21.221	23.231	2.010	CE7	21.221	31.760	10.539	U
23	CE8	22.657	24.687	2.030	CE8	22.657	30.460	7.803	U
24	CE9	22.766	24.675	1.909	CE9	22.766	29.960	7.194	U
25	CE10	21.898	24.564	2.666	CE10	21.898	31.850	9.952	U
26	CE11	21.223	23.223	2.000	CE11	21.223	30.890	9.667	U
27	CE12	20.823	23.778	2.955	CE12	20.823	30.670	9.847	U
28	CE13	20.112	22.123	2.011	CE13	20.112	31.540	11.428	U
29	CE14	20.667	23.987	3.320	CE14	20.667	30.420	9.753	U
30	CE15	22.298	24.170	1.872	CE15	22.298	27.190	4.892	U
			Average	2.332			Average	8.388	

U=Unmethylated

unmethylation, and 6 samples showed both methylation and unmethylation (Col X).

6 samples that showed both methylation and unmethylation were considered to be methylated as done in a similar study.[4] The methylated value (5.990) increased when compared with a unmethylated value (9.189), which suggests that majority in Group B (OSMF patients) is methylated.

Table 4 shows methylation status of p16 in salivary samples of Group A by comparing the target gene p16 (Col IV and VII) with reference gene β-actin (Col III and VI) in the unmethylated and methylated primers, respectively. The average Delta Ct value of unmethylated p16 gene was 6.214 and methylated 12.785. All 30 samples (100%) were unmethylated, and the results suggest that Group A (controls) is unmethylated (Col IX).

Table 5 shows methylation status of p16 in salivary samples of Group B by comparing the target gene p16 (Col IV and VII) with reference gene β-actin (Col III and VI) in the methylated and unmethylated primers, respectively. Out of 30 samples tested 10 were methylated, 15 unmethylated, and 5 showed equal methylation and unmethylation (Col IX). Five samples which showed both methylation and unmethylation were considered to be methylated as done in an earlier study.[4] Since, there was no significant difference between the unmethylated and methylated values the average delta Ct value of Group B (OSMF patients) is considered to be partially methylated.

Discussion

The current study was done to quantitatively evaluate the hypermethylation status of p16 gene in 30 patients with oral submucous fibrosis (OSMF) and to compare it with 30 healthy individuals in their buccal cells and saliva. As the incidence rate of this condition in India is about 5 million people (0.5%) of the total population[3] and as this precancerous condition possesses a risk of 7.6% for malignant transformation over a period of 17 years,[17] this study was designed to investigate an objective marker in early carcinogenesis.

Various studies have evaluated the genetic alteration where there is a progression of normal cells to precancer and cancer.[18,19] A change in gene expression is evident in cancer cells at the epigenetic level via transcriptional inactivation.[6] These epigenetic changes have been identified as an important component of carcinogenesis.[5] DNA methylation is the most important epigenetic alterations that lead to altered gene expression.[6] An increasingly recognized epigenetic mechanism of transcription activation of tumor suppressor genes or DNA repair genes is methylation of

Table 3: Methylation status of p16 in the buccal cells from exfoliative cytology samples of Group B (OSMF patients)

Serial number (Col I)	Unmethylated primer				Methylated primer				
	Sample name (Col II)	β-actin (Ct) (Col III)	Unmethylated (Ct) (Col IV)	Delta (Ct) (Col V)	Sample name (Col VI)	β-actin (Ct) (Col VII)	Methylated (Ct) (Col VIII)	Delta (Ct) (Col IX)	Result (Col X)
1	PE16	21.645	30.16	8.515	PE16	21.645	24.120	2.475	M
2	PE17	21.876	27.89	6.014	PE17	21.876	30.150	8.274	U
3	PE18	20.786	31.93	11.144	PE18	20.786	25.730	4.944	M
4	PE19	20.290	29.78	9.490	PE19	20.290	29.650	9.360	B
5	PE20	21.654	31.56	9.906	PE20	21.654	27.790	6.136	M
6	PE21	21.675	32.78	11.105	PE21	21.675	27.010	5.335	M
7	PE22	21.897	31.89	9.993	PE22	21.897	24.320	2.423	M
8	PE23	20.806	30.43	9.624	PE23	20.806	23.590	2.784	M
9	PE24	20.896	29.34	8.444	PE24	20.896	29.111	8.215	B
10	PE25	21.232	26.34	5.108	PE25	21.232	33.180	11.948	U
11	PE26	22.676	32.76	10.084	PE26	22.676	26.910	4.234	M
12	PE27	20.124	33.49	13.366	PE27	20.124	24.040	3.916	M
13	PE28	21.789	31.52	9.731	PE28	21.789	25.050	3.261	M
14	PE29	22.156	30.13	7.974	PE29	22.156	26.930	4.774	M
15	PE30	21.568	28.98	7.412	PE30	21.568	29.120	7.552	B
16	PE1	21.241	32.14	10.899	PE1	21.241	28.140	6.899	M
17	PE2	20.897	30.47	9.573	PE2	20.897	27.320	6.423	M
18	PE3	22.676	32.150	9.474	PE3	22.676	26.940	4.264	M
19	PE4	21.908	28.650	6.742	PE4	21.908	29.150	7.242	B
20	PE5	22.247	29.867	7.620	PE5	22.247	27.920	5.673	M
21	PE6	23.423	32.150	8.727	PE6	23.423	28.950	5.527	M
22	PE7	21.567	30.450	8.883	PE7	21.567	27.640	6.073	M
23	PE8	20.236	30.710	10.474	PE8	20.236	26.160	5.924	M
24	PE9	20.232	31.640	11.408	PE9	20.232	27.160	6.928	M
25	PE10	20.986	31.610	10.624	PE10	20.986	28.020	7.034	M
26	PE11	21.177	29.430	8.254	PE11	21.177	29.887	8.711	B
27	PE12	20.545	30.546	10.001	PE12	20.545	28.090	7.545	M
28	PE13	22.204	28.640	6.436	PE13	22.204	28.920	6.716	B
29	PE14	23.565	30.890	7.325	PE14	23.565	27.900	4.335	M
30	PE15	20.346	31.654	11.308	PE15	20.346	25.120	4.774	M
			Average	9.189			Average	5.990	

U=Unmethylated, M=Methylated, B=Both methylation and unmethylation, OSMF=Oral submucous fibrosis

normally unmethylated CpG islands in gene promoter region.[20]

p16 is a tumor suppressor gene, normally block cellular proliferation by binding to complexes of cyclin dependent kinase CDK4 and CDK6. This binding prevents entry into the S phase of cell cycle. In many human cancers including oral cancers, frequent loss of p16 is observed. According to Reed *et al*.,[11] p16 expression is lost in 83% of oral cancers and 60% of premalignant lesions, suggesting that p16 alteration is an early event in oral cancer progression.[17] In a similar study by Takeshima *et al*.[4] has shown 70% hypermethylation of p16 gene in OSMF patients in Srilanka. Even though hypermethylation of tumor suppressor genes in malignant tumors including oral cancer has been well documented, very few studies are available on hypermethylation of p16 in oral precancerous conditions. Since, the detection of hypermethylation in precancer can predict the risk of malignant transformation, this study evaluated p16 hypermethylation in OSMF patients.

Sources of free DNA includes serum, plasma, saliva/oral rinse, urine,[21] and cell collection through scraping of the oral mucosa.[14] Compared to serum, saliva has a significant diagnostic advantage as a diagnostic fluid as its collection is noninvasive and simple. According to Righini *et al*.,[22] malignant cells collected in body cavity fluids at direct contact with the tumor shows gene methylation; therefore, salivary analysis has been used in this study for early detection of oral cancer. The advantage of swish methods is higher average DNA yields and longer DNA fragments. According to Mehrotra *et al*.,[23] buccal cell collection through cytobrush technique is a simple, relatively inexpensive, and risk-free method for obtaining cell samples. These advantages justify that salivary rinse and cytobrush technique can be used as an easily accessible method of DNA collection. In this study, salivary rinses were collected by modified method[14] as from the protocol described by Carvalho *et al*.[24] and the buccal samples through cytobrush technique.[14]

Takeshima *et al*.[4] evaluated the hypermethylation status of p14, p15, and p16 in various oral precancers by immunohistochemical methods and has inferred that all frequencies were high in OSMF patients. In this study, hypermethylation of p16 was quantified in salivary rinse and buccal cells of OSMF patients and compared with healthy individuals. Exfoliative cytology samples of Group A (controls) showed no methylation and all 30 samples were unmethylated (100%), whereas in Group B (OSMF patients), 93.3% were methylated, and 6.7% were unmethylated, which suggests that hypermethylation

Table 4: Methylation status of p16 in the salivary samples of Group A (controls)

Serial number (Col I)	Unmethylated primers				Methylated primers			Results (Col IX)
	Sample name (Col II)	β-actin (Ct) Col III	Unmethylated (Ct) (Col IV)	Delta Ct (Col V)	β-actin (Ct) (Col VI)	Methylated (Ct) (Col VII)	Delta Ct (Col VIII)	
1	CS1	20.746	26.780	6.034	20.746	32.403	11.658	U
2	CS2	21.837	28.547	6.710	21.837	34.241	12.404	U
3	CS3	20.127	28.331	8.205	20.127	32.984	12.858	U
4	CS4	20.256	28.986	8.730	20.256	35.436	15.180	U
5	CS5	22.846	27.420	4.574	22.846	34.524	11.678	U
6	CS6	20.621	26.340	5.719	20.621	33.960	13.339	U
7	CS7	20.127	28.982	8.856	20.127	33.007	12.880	U
8	CS8	20.868	28.120	7.252	20.868	33.646	12.778	U
9	CS9	20.121	27.870	7.749	20.121	34.568	14.447	U
10	CS10	22.342	28.465	6.124	22.342	33.657	11.316	U
11	CS11	21.676	26.687	5.011	21.676	32.244	10.568	U
12	CS12	21.124	26.782	5.658	21.124	34.998	13.874	U
13	CS13	21.576	28.781	7.205	21.576	34.193	12.617	U
14	CS14	22.185	27.988	5.803	22.185	33.921	11.736	U
15	CS15	20.757	26.909	6.152	20.757	33.356	12.599	U
16	CS16	20.454	28.720	8.266	20.454	32.454	12.001	U
17	CS17	21.679	28.160	6.481	21.679	33.900	12.221	U
18	CS18	22.547	26.891	4.344	22.547	35.673	13.126	U
19	CS19	21.454	27.165	5.712	21.454	33.876	12.422	U
20	CS20	23.565	27.640	4.075	23.565	34.981	11.416	U
21	CS21	20.787	28.112	7.326	20.787	34.256	13.470	U
22	CS22	20.214	28.154	7.940	20.214	34.099	13.885	U
23	CS23	21.345	25.820	4.475	21.345	34.899	13.554	U
24	CS24	20.676	26.341	5.665	20.676	35.908	15.232	U
25	CS25	20.239	27.140	6.901	20.239	33.256	13.018	U
26	CS26	20.925	26.190	5.266	20.925	33.872	12.947	U
27	CS27	21.566	27.000	5.434	21.566	34.990	13.425	U
28	CS28	23.454	27.232	3.778	23.454	35.534	12.081	U
29	CS29	22.786	26.530	3.744	22.786	33.165	10.380	U
30	CS30	20.908	28.123	7.216	20.908	35.354	14.447	U
			Average	6.214		Average	12.785	

U=Unmethylated

of p16 is very highly significant in buccal cell samples and is consistent with previous studies.[25-27] Although the unmethylated percent (6.7%) in the buccal cell samples are negligible, it can be attributed to sampling and storage/ processing errors.

According to Lee *et al.*,[28] hypermethylation rate can be low in salivary rinse due to the dilution effect of normal-unmethylated genomes present from normal mucosa. However, in the present study, results from the salivary rinse shows that 50% of Group B are methylated, and 50% are unmethylated. No methylated samples were present in Group A (control), and all 30 samples (100%) were unmethylated. These results suggest that percentage of methylation and unmethylation is same, and Group B is considered to be partially methylated, which is consistent with previous studies.[4]

Comparison of the methylation status of p16 between buccal cells and salivary samples of Group B shows that 22 samples in buccal cells were methylated, 2 samples were unmethylated, and 6 samples were both unmethylated and methylated. Among the salivary samples, 10 were methylated, 15 were unmethylated, and 5 samples were both unmethylated and methylated, which infers that the hypermethylation of p16 was significantly higher in buccal cell samples than the salivary samples and consistent with earlier studies.[25-30] Statistical analysis to indicate the *P* value through odds ratio was not done in this t study as Group A was completely unmethylated (0%).

Conclusion

To conclude significant quantity of p16 hypermethylation were present in buccal cells and saliva. Buccal cell sampling may be a better sampling method for evaluation in this study than the salivary samples. However, the quantification of p16 hypermethylation in oral cancer group as a part of the study may lead to a definitive conclusion about the transformation of precancer to cancer and will serve as a very early indicator of carcinogenic activity and aid in the prognosis of the disease.

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Table 5: Methylation status of p16 in the salivary samples of Group B (OSMF patients)

Serial number (Col I)	Methylated primers				Unmethylated primers			Results (Col IX)
	Sample name (Col II)	β-actin (Ct) (Col III)	Methylated (Ct) (Col IV)	Delta (Ct) (Col V)	β-actin (Ct) (Col VI)	Unmethylated (Ct) (Col VII)	Delta (Ct) (Col VIII)	
1	PS1	21.645	26.930	5.285	21.645	32.787	11.142	M
2	PS2	20.896	29.036	8.140	20.896	28.228	7.332	B
3	PS3	21.232	27.213	5.981	21.232	29.980	8.748	B
4	PS4	21.876	28.020	6.144	21.876	28.354	6.478	B
5	PS5	20.786	28.100	7.314	20.786	32.765	11.979	M
6	PS6	21.568	29.823	8.255	21.568	28.756	7.188	B
7	PS7	21.897	27.827	5.930	21.897	31.987	10.090	M
8	PS8	21.908	29.671	7.763	21.908	28.877	6.969	B
9	PS9	21.654	32.670	11.016	21.654	26.176	4.522	U
10	PS10	22.156	26.357	4.201	22.156	32.403	10.247	M
11	PS11	20.806	31.573	10.767	20.806	26.531	5.725	U
12	PS12	22.676	31.492	8.817	22.676	26.778	4.102	U
13	PS13	20.124	33.122	12.998	20.124	25.037	4.913	U
14	PS14	21.789	31.051	9.262	21.789	25.986	4.197	U
15	PS15	21.241	26.746	5.505	21.241	31.971	10.730	M
16	PS16	23.423	26.818	3.395	23.423	33.999	10.576	M
17	PS17	20.290	32.486	12.196	20.290	27.576	7.286	U
18	PS18	20.986	27.540	6.554	20.986	32.898	11.912	M
19	PS19	22.676	32.113	9.437	22.676	25.942	3.266	U
20	PS20	21.177	26.245	5.069	21.177	31.453	10.277	M
21	PS21	22.247	31.178	8.931	22.247	26.900	4.653	U
22	PS22	21.567	30.109	8.542	21.567	24.167	2.600	U
23	PS23	22.204	26.453	4.250	22.204	34.908	12.705	M
24	PS24	20.236	30.287	10.051	20.236	28.432	8.196	U
25	PS25	21.675	31.004	9.329	21.675	25.570	3.895	U
26	PS26	20.545	30.818	10.273	20.545	27.982	7.437	U
27	PS27	20.346	25.130	4.784	20.346	33.615	13.269	M
28	PS28	23.565	31.902	8.337	23.565	26.543	2.979	U
29	PS29	20.897	35.670	0.000	20.897	28.400	7.503	U
30	PS30	20.232	31.982	11.750	20.232	28.244	8.012	U
			Average	7.676		Average	7.631	

U=Unmethylated, M=Methylated, B=Both methylation and unmethylation, OSMF=Oral submucous fibrosis

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Conflicts of interest

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