Original Article

Microsatellite instability in D2S123 flanking the *hMSH2* gene in oral squamous cell carcinoma in South India

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ABSTRACT

Background: Oral cancer is the third most common cancer in India. It is a multifactorial disease. Cells with defective mismatch repair gene *hMSH2* can result in genomic instability in oral squamous cell carcinoma (OSCC) tumors. The objective of this study was to investigate the incidence of microsatellite instability (MSI) flanking the *hMSH2* in OSCC tumors and relate the MSI status with the mutation profile of *hMSH2* in Malayalam speaking population from Kerala. **Materials and Methods:** Patients diagnosed with OSCC, without superimposed premalignant and other malignant conditions, were recruited for the study based on strict selection criteria. 37 subjects from Malayalam speaking ethnic background of Kerala in India were selected. Blood and carcinoma tissues from were obtained from each patient diagnosed with OSCC. Big adenine tract 26 (BAT26) and D2S123 microsatellite flanking the *hMSH2* gene were assessed for their peak patterns in each patient's blood and tissue DNA to analyze MSI and loss of heterozygosity (LOH). **Results:** No MSI was observed in any of the patient at BAT26 loci. Though BAT26 is reported to be quasi monomorphic, but interestingly it was found to be polymorphic in one patient. In D2S123 loci microsatellite alterations (MA) were observed in 50% of the OSCC patients, which comprised of both LOH and MSI. MA was observed to be significantly increased in moderately differentiated OSCC tumors. These MSI and LOH were independent of clinicopathological characteristics and mutation profile of *hMSH2*. **Conclusion:** Higher incidence of MSI at D2S123 in OSCC tumors could be indicative of diagnostic significance. However, this needs to be validated further in increased sample size and across different ethnic population.

Key words: Big adenine tract 26, D2S123, human mismatch repair (*hMSH2*) gene, oral squamous cell carcinoma

INTRODUCTION

Neoplastic transformation in the normal human cell occurs as a result of a series of genetic alterations, including the loss, gain, or amplification of different chromosomes.[1] These multiple genetic changes gradually transform growth-limited cells into highly invasive cells, unresponsive to growth controls. Genes that positively control cell cycle checkpoints are the targets for oncogenic activation in cancer, whereas negative regulators, such as tumor suppressor genes, are targeted for inactivation.[2] The genetic evolution of normal cells into cancer cells is largely determined by the fidelity of DNA replication, repair, and division.[3] Human mismatch repair genes (hMMR) have the ability to repair both mismatched bases and insertion loop errors during DNA replication. MMR genes involve a set of genes including, but not limited to *hMLH1*, *hMSH2*, *hMSH6*, *hPMS2*, *hMSH3*, and *hPMS1*.[4,5] Suboptimal DNA repair could result in disrupting the pattern of repeat sequences, causing chromosomal aberrations in the genome of patients suffering from instability syndromes. Length alterations in mono-, di-, tri-, tetra-, and pentanucleotide repeat in the MMR genes are termed as microsatellite instability (MSI). Microsatellite alteration (MA) is characterized by MSI and loss of heterozygosity (LOH). Significant proportions of carcinomas develop through DNA MMR deficiency and

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exhibit frequent MA.[6-9] A study conducted in India in 2010 showed that oral cancer is the most common cancer in men aged 30-69 years, and fourth most common in women aged 30-69 years.[10] There have been few inconsistent reports regarding the role of microsatellites flanking human MMR genes in oral squamous cell carcinoma (OSCC).[11,12]

The South Indian state, Kerala, with high incidence of oral cancer,[13] is an ideal setting for studies on oral cancer. In an earlier study, we have reported that polymorphic variants in the MMR gene *hMSH2* can result in OSCC in Malayalam speaking South Indian population and could indicate defective repair mechanism or MSI. With this intention, we have tried to decipher the role of microsatellites flanking *hMSH2* in oral cancer and the correlation of these with clinicopathological parameters in patients from South India. In the present study, we aim to investigate the MAs in big adenine tract 26 (BAT26) and D2S123 flanking the *hMSH2* gene in OSCC tissues and its corresponding blood from same patients. MSI status will be further assessed for background mutation profile of *hMSH2* gene.

MATERIALS AND METHODS

A prospective study based design was conducted for 1 year. Based on strict selection criteria, 37 subjects from Malayalam speaking ethnic background of Kerala in India were selected. Patients diagnosed with OSCC, without superimposed premalignant and other malignant conditions, were recruited. A proforma was designed to record socio demographic details such as: Name, age, gender, habits, site, and histopathological grading. The study was approved by the Institutional Ethical Committee of Government Dental College, Trivandrum. Peripheral blood sample (3 ml) was collected in EDTA vials and stored at −70°C till DNA isolation. After the biopsy of the tumor tissue, half of the tissue was fixed in formalin and sent for histopathology and remaining half was immediately stored at −70°C for further use in DNA studies. The tissue samples were included in the study after histopathological confirmation of OSCC. Thus, both blood and carcinoma tissues from all 37 subjects were obtained. DNA was extracted using conventional phenol chloroform method. The concentration of the genomic DNA isolated from each blood sample was checked by measuring its absorbance at 260 nm using a spectrophotometer (BioSpec-1601, Shimadzu). The ratio of absorbance at 260 nm and 280 nm (A260/A280) was used to estimate the purity of DNA. A260/A280 between 1.7 and 1.8 indicated good quality DNA, relatively free from protein contamination. Primers for BAT26 and D2S123 flanking the *hMSH2* were designed and synthesized with a 6-FAM labeled fluorescent dye for the forward primer. Markers used in the study with their primer sequences and their chromosomal location are presented in Table 1. PCR was set with primers, AmpliTaq Gold Taq polymerase and ABI PRISM True allele PCR Premix. Monoplex PCR was done to increase the sensitivity of the microsatellite assay. Amplification for BAT26 was performed with initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C 45 s, annealing at 57°C for 45 s, extension at 74°C for 45 s and final extension at 74°C for 7 min. Similarly, amplification for D2S123 was performed with initial denaturation at 95°C for 12 s followed by 30 cycles of denaturation at 94°C 30 s, annealing at 57°C for 45 s, extension at 72°C for 60 s and final extension at 72°C for 5 min. The amplified fragments were then resolved by capillary electrophoresis using an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) and the fluorescent data were collected during fragment separation by GeneScan analysis. DNA size standards were used in the separation and scanning of the fragments. Raw data was analyzed with the autobinning option using GeneMapper software v4.0 (Applied Biosystems, Foster City, CA, USA), which has user adjustable parameters such as analysis range, peak detection settings, size range of peaks to be tabulated, and size calling method. The automatic allele calls were confirmed visually by examining the GeneScan electropherograms. Peak patterns of the electropherogram in each patient's blood and tissue DNA were compared with analyze MSI and LOH. Microsatellites isolated from blood samples were considered to be normal, and alterations in the microsatellites of tissue were studied. Mutation analysis of the selected single nucleotide polymorphism rs2303428 (*hMSH2*–6C/T) located at −6 of the 3′ splice acceptor site of exon 13 has been described earlier.

Loss of heterozygosity

Allelic loss was scored if there was a complete loss of one allele or if the relative peak intensity of one allele was decreased by at least 50% in the tumor, compared with the same allele in the corresponding normal tissue. The value was calculated using a normalized allele ratio (LOH ratio) equation in which R = (A1) (N2)/(A2)(N1) where A1 and A2 are the heights of the alleles from DNA of biopsy sample and N1 and N2 are the heights of the alleles from the DNA from blood. An LOH index of >1.5 (loss of the smaller allele) or <0.67 (loss of the large allele) was considered to be least 50% reduction in relative peak intensities. Therefore, cases in which the LOH ratio was ≤0.67 or ≥1.5 were scored as LOH. For the calculation of LOHs, samples showing homozygosity were not considered.

Microsatellite instability

Microsatellite instability was scored if one or both alleles at a given locus showed size variation, that is, either expansion or contraction or by the presence of novel peaks in comparison with that of normal tissue. Stutter peaks were carefully considered. Tumors were classified as microsatellite stable (MSS) if no alteration was found. Statistical analysis on

Table 1: Markers used in the study with their primer sequences and their chromosomal location

Microsatellite markers	Locus	Position in locus	Product length	Primer sequence	Temperature (°C)
BAT26 (A) 26	2p21–22	Within intron 5 of *hMSH2* gene	116	F5'-TGA CTA CTT TTG ACT TCA GCC-3' R5'-AAC CAT TCA ACA TTT TTA ACC C-3'	57
D2S123 (CA) 28	2p16	Proximal to MSH2 gene	194–227	F5'-TCG CCT CCA AGA ATG TAA GT-3' R5'-TCT GCA TTT TAA CTA TGG CTC-3'	57

BAT26: Big adenine tract 26

quantitative data was undertaken using Chi-square analysis or Fisher's exact test wherever appropriate. $P < 0.05$ was considered as statistically significant.

RESULTS

Demographic variables of the study are presented in Table 2. The patient population comprised of 21 males (57%) and 16 females (43%) who fell in two age groups, 26 subjects were above 60 (70%) and 11 were below 60 (30%). Data on habits show 22 patients with tobacco chewing habit (59%), 11 patients with tobacco smoking habit (30%) and four patients were without tobacco habit (11%). Only 6 patients were consuming alcohol (16%). Fourteen tumors were in the buccal mucosa (38%), 14 were in the tongue (38%), two each in the retro molar area and alveolus (5% and 5%), one each in the soft palate and hard palate and three in the gingiva. Fourteen were well-differentiated (38%) and 23 were moderately differentiated (62%). We screened 37 samples for MSI at BAT26 locus. Though BAT26 is reported to be quasi monomorphic, interestingly in one patient subject BAT26 was found to be polymorphic [Figure 1]. In this patient subject, the size of the shorter allele was 106 bp and longer allele was 116 bp in both blood and tissue DNA. However, no MSI was observed in this individual. No MSI was observed in any of the patient at BAT26 loci.

Similarly for D2S123 we screened 37 samples for MAs wherein, 5 were noninformative [Table 3]. Interestingly among remaining 32 samples, 16 showed MAs of which 6 showed MSI [Figure 2a-e], 8 showed LOH [Figure 2f] and two had both LOH and MSI. This indicated that 50% of the OSCC patients displayed MA at D2S123. MSIs were in the form of monoallelic increased shift [Figure 2a], biallelic increased shift [Figure 2b], biallelic decreased shift [Figure 2c], increase in peak shift of one allele with decrease in other allele [Figure 2d] and appearance of novel peak when compared to homozygous blood counterpart [Figure 2e].

While correlating the MA with the histopathological differentiation of OSCC, we observed that 91% of the moderately differentiated tumors have MA whereas only 21% of the well-differentiated tumors showed MA[Table 3]. Thus, MA was observed to be significantly increased in moderately differentiated OSCC. Similarly when clinicopathological characteristics such as age, gender, tobacco and site, were assessed for MA we did not observe any significant difference. We further assessed the mutation profile rs2303428 (*hMSH2*–6C/T) located at −6 of the 3' splice acceptor site of exon 13 of the MMR gene *hMSH2* in both microsatellite altered and MSS OSCC tumors. We could not find any significant difference in the mutation profile of *hMSH2* gene between microsatellite altered and MSS OSCC tumors [Figure 3].

Table 2: Demographic details of the study group

Demographic Information	Number	Percentage
Sample size		
OSCC patients blood	37	
OSCC patients biopsy tissue	37	
Gender		
Male	21	57
Female	16	43
Age		
≤60	11	30
>60	26	70
Tobacco usage		
Smoking	11	30
Chewing	22	59
No habit	4	11
Alcohol consumption		
Yes	6	16
No	31	84
Histopathological differentiation		
Poor	5	13
Moderate	18	49
Well-differentiated	14	38

OSCC: Oral squamous cell carcinoma

Table 3: Distribution of MAs (MIN and LOH) of BAT26 and D2S123 in OSCC cases

Nature of alteration	BAT26	D2S123
MSS	34 (97)	16 (50)
MAs	1 (3)	16 (50)
MSI	1	6
LOH	0	8
MSI+LOH	0	2
No result	2	5
Total OSCC cases	37	37
Histopathology		
Moderately differentiated		13
Well differentiated		3

Frequencies are mentioned in brackets. MSI: Microsatellite instability, LOH: Loss of heterozygosity, OSCC: Oral squamous cell carcinoma, BAT26: Big adenine tract 26, MSS: Microsatellite stable, MAs: Microsatellite alternations

DISCUSSION

Genomic instability is a broad name which includes chromosomal instability and MAs. MAs could be explained

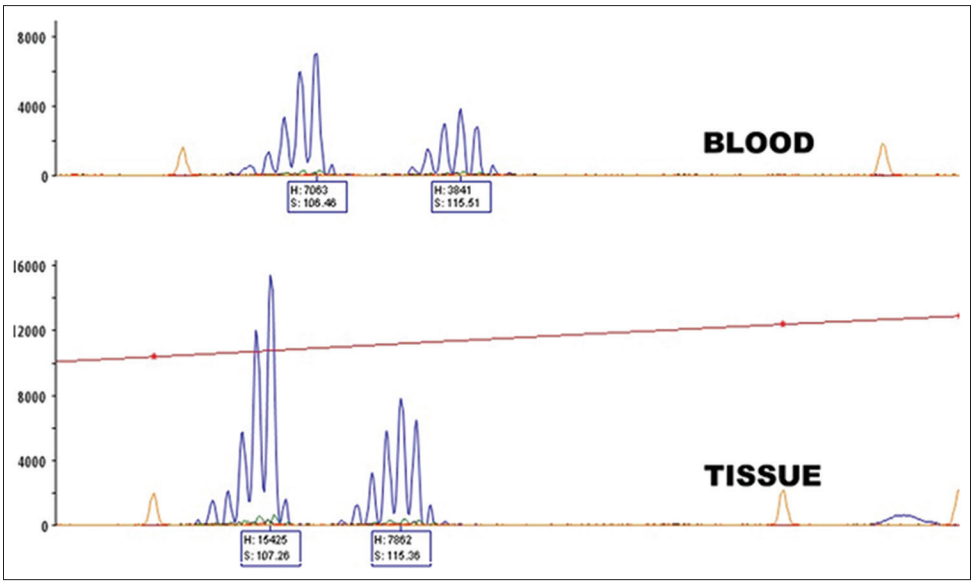

Figure 1: Presence of allele 106 and 116 at big adenine tract 26 indicating polymorphism at this locus

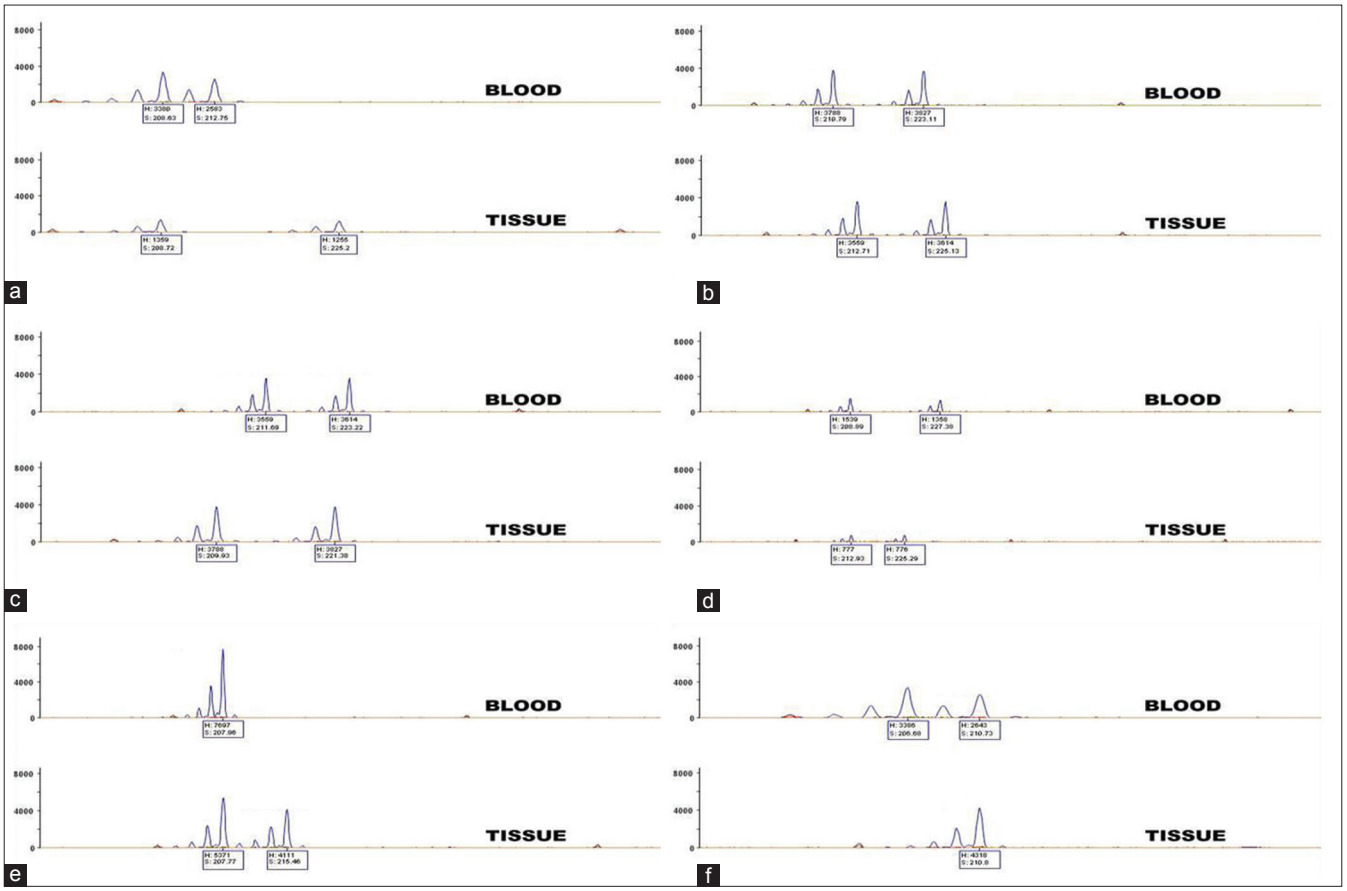

Figure 2: Patterns of microsatellite alterations in oral squamous cell carcinoma patients from South India. (a) Monoallelic increased shift (b) biallelic increased shift (c) biallelic decreased shift (d) increase in peak shift of one allele (e) appearance of novel peak (f) loss of heterozygosity at D2S123

on the basis of defective MMR process. In an earlier study, we have reported that polymorphic variants in the MMR gene *hMSH2* can result in OSCC in Malayalam speaking South Indian population and could indicate defective repair mechanism or MSI. In the present study, we were keen to understand whether these variants in *hMSH2* translate to MAs.

Microsatellite markers flanking the *hMSH2* gene, BAT26 and D2S123 were assessed in blood and OSCC tumors of

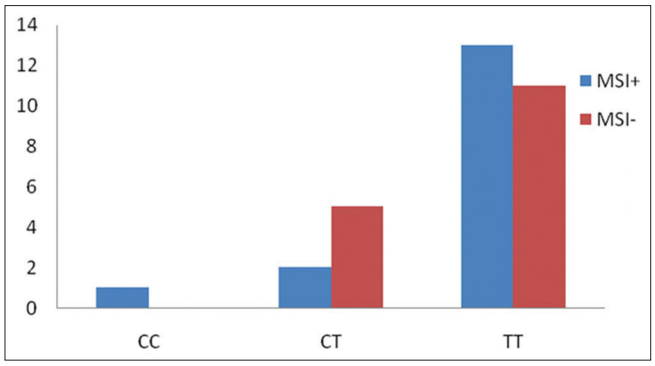

Figure 3: Mutation profile of rs2303428 (*hMSH2*–6C/T) of exon13 of *hMSH2* in microsatellite altered (MSI+) and microsatellite stable (MSI−) oral squamous cell carcinoma tumors

each patient. There was no MSI observed in BAT26 in any of the OSCC tissues. Careful analysis of published data on head and neck squamous cell carcinoma reveals that similar results were also obtained by Nunn *et al*.,[14] Chakrabarti *et al*.[15] and Wang *et al*.[16] with exception to Demokan *et al*.[17] who reported a significant association of MSI at BAT26 in oral cancer using polyacrylamide gel electrophoresis. The observation in our study contradicts Demokan's finding. The capillary electrophoresis method, which was used in our study had the distinct advantage over the conventional standard autoradiographic analysis and polyacrylamide gel electrophoretic analysis[18] done by Demokan *et al*.[17] Hence, this present study is in concordance with majority of the studies that might have accurately assessed the frequency of MSI in BAT26.

According to Hoang *et al*.[19] BAT26 is a quasi-monomorphic marker. Though the aim of the study was not to understand the distribution of allelic pattern in the population, but interestingly in the present study we report the presence of polymorphism in BAT26. This polymorphism in BAT26 was observed in a single individual in both blood as well as in tumor tissue. This rare allele was not a result of MSI at BAT26. There is only one study conducted by Samowitz *et al*.[20] reporting a second, significantly shorter allele of BAT26 (106 bp) that suggested that a germline polymorphism exists for BAT26 in 7.7% of African-Americans and 0.08% of Caucasians. However, existence of germline polymorphism in BAT26 was contradicted by Bradshaw *et al*.[21] who drew attention to the possibility of methodological errors leading to the observation of this shorter allele. They suggested that constitutional DNA analyzed for BAT26 with a fluorescently labeled PCR primer using the ABI 377 (PE Applied Biosystems, Warrington, UK), occasionally gives rise to a trace suggesting the presence of a shorter allele. Moreover, in both of the traces obtained with BAT26 primers on constitutional DNA shown by Samowitz *et al*.[20] the fluorescence signal is less than 150 relative units. According to PE Applied Biosystems, under 150 units "the signal-to-noise ratio is too low to discriminate between sample peaks and background." In concordance with Samowitz *et al*.,[20] the size of the shorter allele in our finding was 106 bp and longer allele was 116 bp in DNA of blood and tissue. In both, the tumor tissue and blood the electropherogram of the shorter allele as observed in ABI 3730 capillary electrophoresis machine, showed peak height to be more than 3000 rfu. Thus, we conclude that there is no space for error in reporting the presence of this novel and rare allele.

Duval and Hamelin[22] suggested that MA in BAT26 can be characterized without the need for matched, normal tissue DNA for comparison. However, considering the prevalence of rare shorter allele of BAT26 in such a small sample size of our study population, we may suggest that routine evaluation of both normal as well as tumor specimens in every patient should be advised to avoid mistakes in referring MA.

In contrast to BAT26 that has been investigated extensively and is being recommended for routine evaluation of MA we observe greater incidence (43.2%) of MA at D2S123. As D2S123 is heterozygous, the present microsatellite assay revealed alterations compatible with two different mechanisms, LOH and MSI. Out of MSI positive cases, several types of alterations seen in this experiment have already been reported by Chakrabarti *et al*.[15] in context to other markers. Due to the inbreeding tendency[23] there is an increased number of homozygous conditions in polymorphic loci in Indian population. For this reason, there were very limited numbers of patients with heterozygosity in our study. The present study could not associate MA with any clinicopathologic findings except a statistically significant correlation with the histopathological grading ($P < 0.05$). MA is more frequent in moderately differentiated squamous cell carcinoma than well-differentiated squamous cell carcinoma.

In the present study, we report a high incidence of MA in OSCC patients at D2S123 loci in South India. It is only fair to suggest that MA at D2S123 may lead to OSCC cancer, but its role in prognosis could not be substantiated due to lack of association with histopathological grading. In an earlier study by Choudhary *et al*.[24] we reported that mutations in *hMSH2* may result in MSI. In the present study, we could not correlate the mutation in *hMSH2* with MAs or with histopathological grading. Several studies have indicated the role of MMR genes in OSCC[9,14,16] where the authors have suggested that MMR gene inactivation is rare among primary HNSCC. On the other hand, Demokan *et al*.[17] has contradicted these findings and suggested that instability at the BAT26 and D2S123 loci were associated with the MSI-high status. These conflicting data on the role of microsatellites may be due to lack of uniform criteria of evaluation and differing MSI detection techniques.

Various investigators have elaborated on two important criteria, that is, micro-dissection of cancer cells and use of monoplex PCR, to resolve the MA in cancer tissues.

Danjoux *et al*.[25] evaluated the impact of contaminant normal DNA in tumor samples for LOH and MSI assessment in colorectal cancer with and without the micro-dissection technique in 23 cases of colorectal cancer using fluorescent polymerase chain reaction. They observed an increase of more than 60% in the LOH detection rate after micro-dissection whereas they emphasized that omitting the micro-dissection has not adversely affected the MSI assessment. Therefore, we conclude that the frequency of LOH might be under-evaluated due to the omission of micro-dissection technique in our study but the assessment of MSI could be free of error. Another distinct advantage of the study is the use of monoplex fluorescent PCR reactions followed by monoplex detection using fluorescent based electrophoregram detection system. The monoplex fluorescent PCR reactions are reported to have enhanced sensitivity.[18]

A major advantage of the fluorescence-based MSI and LOH analysis is the precise determination of loss of alleles by calculation of the ratio of the peak heights of normal and tumor alleles using GeneScan software. The modern automatic fragment analysis procedures offer more precise and quantitative assessment of MSI and LOH in comparison to gel electrophoresis methods.[26,27] Therefore, it could be concluded that the results of the present study might indeed devoid of error.

CONCLUSION

The present study identifies increased incidence of MAs in D2S123 in OSCC patients. However, this MA was found to be independent of histopathological grading and *hMSH2* mutation profile in OSCC. The study also reports the presence of polymorphism in BAT26, which was independent of OSCC status.

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