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Expression of Mismatch Repair Protein Mlh1 in Primary Oral Squamous Cell Carcinoma in An Indian Population – An Immunohistochemical Study

KEYWORDS: DNA mismatch repair, carcinoma, squamous cell, immunohistochemistry

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ABSTRACT *Background: MutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli), also known as MLH1 is an integral DNA mismatch repair (MMR) gene whose role in tumorigenesis has been implicated in an extensive group of human cancers. Our study attempted to correlate MLH1 immunoexpression with different clinicopathological parameters in oral squamous cell carcinoma (OSCC) by immunohistochemical staining.*

Materials and method: A retrospective cross-sectional study was carried out to detect MLH1 in formalin fixed paraffin embedded (FFPE) specimens of OSCC (n=60) by immunohistochemistry.

Results: Positive nuclear expression of MLH1 in tumor cells was recorded and scored. An over expressed MLH1 in well differentiated OSCC cases with a significant reduction in its expression with deteriorating histologic grade was observed ($P<0.001$). Also, the MLH1 immunoexpression was directly proportional to the tumor stage ($P<0.05$).

Conclusion: The over expression of MLH1 thus reflects an attempt to amend the DNA lesions through the MMR system and restore genomic stability. Analysis of MLH1 expression may assist in prognostication and aid in designing superior treatment protocols.

Introduction:

Oral squamous cell carcinoma (OSCC) is the sixth most common malignancy and a major cause of morbidity and mortality worldwide(1) Globally, about 500,000 new cases of oral and pharyngeal cancers are diagnosed annually and three-quarters of these are from the developing countries with India alone reporting around 65,000 (2). In India, the age standardized incidence rate of oral cancer is 12.6 per 100,000 population (3). Although no single causative agent or factor is attributable to the genesis of OSCC, tobacco usage in various forms continues to be an important risk factor.

Cancer occurs through the sequential accumulation of genetic defects, followed by clonal expansion. The DNA mismatch repair (MMR) system is necessary for the maintenance of genomic stability of a cell (4). This system repairs DNA damage related to replication errors including mismatched bases and strand slippage. The mutL homolog1 (MLH1) protein of the colon cancer nonpolyposis type 2 in E coli, a product of the MLH1 gene, is an integral part of the mismatch repair complex. An alteration in this protein expression is associated with the acquisition of a mutator phenotype, microsatellite instability and a predisposition to cancer (5)

A few studies in the head and neck squamous cell carcinoma (HNSCC) and esophageal cancers have reported the presence or absence of MLH1 mutation (6, 7, 8). Our study aimed to assess and quantitate the immunohistochemical expression of MLH1 in OSCC cases by chart analysis. This profile provided us an opportunity to compare it with various clinicopathological parameters and thereby determine the prognostic and therapeutic implications.

Materials & Methods:

Patient population:

A total of 60 patients diagnosed and treated for primary OSCC at Kasturba Hospital, Manipal between 2005 and 2011 were selected for our study. Relevant clinical and follow-up data were obtained through medical records. For each case, age, gender, tumor site, habit history, oral hygiene status, tumor stage (I–IV), primary treatment, histological type and follow-up data was obtained. (Table 1) The tumor staging was done as per the classification proposed by American Joint Committee on Cancer (AJCC, 2006)(9). Cases with insufficient tumor tissue in their paraffin blocks, incomplete clinical information, a previous history of radiotherapy or chemotherapy prior to surgery were excluded from the study. Institutional Ethics Committee approval was obtained (IEC 76/2011) to carry out this study. For analysis, formalin fixed paraffin embedded (FFPE) blocks of OSCC cases retrieved from the archives of the department of oral pathology were histopathologically graded according to the World Health Organization (WHO) classification of tumors(10).

Immunostaining procedure: Four µm sections were taken on APES (3-Aminopropyl triethoxysilane) coated glass slides for immunostaining. Positive tissue control consisted of a sample of normal colon tissue while the adjacent stromal and lymphoid cells with positive nuclear expression in the test sections served as internal positive controls. Negative tissue control consisted of a sample of normal skeletal muscle. The immunostaining (polymeric horseradish peroxidase technique) included antigen retrieval in citrate based buffer (pH 6.0), neutralization of endogenous peroxide with peroxide-block for 5 minutes, protein block for 5 minutes, incuba-

tion with liquid mouse monoclonal primary antibody (NCL-L-MLH1, clone: ES05; Leica Microsystems, UK) diluted 1:100 in PBS dilution for 30 minutes, post primary block for 30 minutes, incubation with secondary antibody for 30 minutes, visualization of sections with diamino benzidine tetra hydrochloride (DAB) working solution for 5 minutes and counterstaining with Mayer's Hematoxylin for 3 minutes followed by dehydration, clearing and mounting. Negative and positive controls were used during each staining run.

Evaluation of staining
Two observers who had no prior knowledge of the cases assessed the staining and expression pattern of MLH1 in each case independently. Assessment of immunostaining was performed using light microscope (Pentahead Microscope: Olympus – Model: U-MDOB3, Tokyo, Japan) at 10X and 40X magnifications. Tumor cells were considered immunopositive if they presented with brown nuclear staining, regardless of their intensity. To obtain representative indexes, 16 high power fields (40X) were analyzed for each slide as per the criteria used by Fernandes, et al (11). Both the positive and negative cells were counted in all fields. Positive cells divided by the total number of cells counted in all fields and multiplying the result with 100 provided the percentage of positive cells for each case. A scoring for MLH1 expression was graded as negative - 0% of positive cells; reduced - < 50% positive cells; normal - 50-75% of positive cell; increased - 75% of positive cell.

Statistical analysis
Statistical analysis was performed using SPSS version 16.0. The kappa statistics for testing the agreement between 2 observers and chi square test to evaluate the association between all the clinicopathological parameters and MLH1 immunoexpression was applied. A p value less than 0.05 was considered statistically significant.

Results
A chart analysis of all the cases showed 21 well differentiated (35%), 20 moderately differentiated (33.3%) and 19 (31.7%) poorly differentiated OSCC. Greater proportion (70%) of OSCC cases was of the advanced stage (Stage III & Stage IV) at the time of diagnosis. The patients' age ranged from 26-84 (mean age 55) years with a definite male predilection of 2.7:1. Buccal mucosa was the most common site of involvement (35%) followed by anterior 2/3rd of the tongue (25%), lower alveolar ridge (18.3%), upper alveolar ridge (10%), retromolar trigone (5%), floor of the mouth (3.3%), lip (1.7%) and hard palate (1.7%). The most common abusive habit recorded in the cases was a combination of tobacco and areca nut chewing (23.3%), followed by areca nut chewing alone (20%). The other predominant habits included combined use of beedi or cigarette smoking and alcohol. The affected individuals who did not have habits constituted a small percentage of cases (6.7%). In addition, the oral hygiene of majority of patients (93.3%) was poor (Table 1). The association of the immunoexpression of MLH1 with the clinicopathological features is given in Table 2.

Out of the 42 cases in advanced stage (Stage III and IV), 20 (33.33%) showed over expression, while 5 out of 18 cases of SCC in the early stage (Stage I and Stage II) showed a reduced MLH1 immunoexpression. With regard to histologic grading, out of the 21 cases of well-differentiated SCC, 19 cases (90.47%) showed an over expression of MLH1. The remaining 2 cases showed a normal expression of the protein (Figure 1.). The immunostaining was confined to the nucleus (Figure 2.) and the staining was particularly strong in tumor cells, which were pleomorphic. The dysplastic epithelial margins stained intensely with MLH1 (Figure 3.). The staining declined with deteriorating grade with almost no staining present in some poorly differentiated OSCC cases (Figure 4.) Out of the 20 cases of moderately differentiated SCC cases 11 (18.3%) showed normal MLH1 expression, 7 (11.7%) showed over expression and remaining 2 cases (3.3%) cases showed a reduced immunoexpression of the MLH1 protein. (Figure 5.) Out of the 19 poorly differentiated SCC cases 5 (8.3%) showed a reduced expression (Figure 6.), 11 (18.3%) revealed normal expression of MLH1 and the remaining 3 cases revealed MLH1 overexpression.

The patient demographics including age, sex, site, habits and oral hygiene status did not show any significant association with MLH1 (Table 2.), while the association of MLH1 with tumor grade and stage reached a significant value. ($P<0.05$)

Discussion
Despite an extraordinary fidelity in DNA synthesis, errors do persists. Such errors can be detected and repaired by post replication mismatch repair system. Defects in DNA could be restricted to a single strand or affect both the strands. When one of the two strands of a double helix has a defect, the other strand could be used as a template to guide the correction of the damaged strand. Failure to accomplish this may lead to cancer.

In order to repair damage, there exist a number of excision repair mechanisms that remove the damaged nucleotide and replace it with an undamaged nucleotide complementary to that found in the undamaged DNA strand. Three types of repair mechanisms exists which include base excision repair, nucleotide excision repair and mismatch repair (MMR)(12). MMR system is necessary for the maintenance of genomic stability. It corrects the errors of DNA replication and recombination which result in mispaired (but undamaged) nucleotides (Figure 7.). Its other functions include correction of biosynthetic errors and DNA damage surveillance. DNA repair enzymes help to ensure the conformity of the genetic code but further genetic changes or mutations are inevitable (13). Defects in MMR result in the accumulation of mutations in tumor suppressor genes and oncogenes leading to initiation of tumorigenesis(14).

MLH1 and MSH2 form the main components of the MMR system. Overexpression of MLH1 and MSH2 induces apoptosis in either repair proficient or deficient cells. Loss of apoptosis as a result of their deficiency may be an important factor in cancer susceptibility (15). The role of MLH1 defects in development of cancer is illustrated in Figure 8. Defects in MLH1 is the primary cause for hereditary non-polyposis colorectal cancer type-2 (HNPCC-2) and cancers in certain other tissues like uterus, ovary, breast, stomach, small intestine, skin and larynx.

Recently, the loss of DNA mismatch repair proteins has been highlighted in HNSCC including OSCC. These studies are however limited to assessing the presence or absence of MLH1 protein in OSCC and its association with microsatellite instability (MSI) and gene mutation. Extrapolating these results, we evaluated the immunoexpression of MLH1 in a series of OSCC cases (n = 60) which were histologically graded and treated at our center. Immunostaining for MLH1 has shown a great sensitivity and specificity in detecting MSI phenotype as it helps to pinpoint the affected gene (16, 17).

Lo Muzio, et al (6) for the first time proposed that absent nuclear staining of MLH1 and MSH2 could mark the potential mutator phenotype for OSCC. Subsequently, Fernandes, et al (11) observed that MLH1 overexpression was associated with well differentiated tumors while a reduction/negative expression was detected in the poorly differentiated counterparts. In a study conducted by Theocharis, et al (18) on 49 tongue squamous cell carcinomas, a high MLH1 expression was reported in association with the presence of lymph node metastases. Also, a high level of the same protein was frequently observed in patients with well-differentiated tumors and those without any evidence of perineural invasion.

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In our study the expression of DNA repair protein MLH1 was predominant in the tumor cells. A decreased to negative expression was noted in the most differentiated layers of epithelium and keratin pearls did not express the protein. A greater proportion of the well-differentiated SCC demonstrated an over expression of MLH1 as compared to the moderately and poorly differentiated SCC. This reflects an exuberant attempt on the part of MMR system to repair the genetic errors in the tumor cells. The reduced expression of MLH1 in poorly-differentiated SCC suggests a possible exhaustion of the MMR system. Previous studies have emphasized the association of loss of expression of DNA repair proteins with poor differentiation and prognosis, reduced postoperative survival, extensive invasion and increased metastatic potential (18) Although an inconsistent association has been noted between the stage of the tumor and MMR protein expression (19,20), the MLH1 immunoexpression was found to be directly proportional to the tumor stage. Hence, it could be anticipated that with advancement of the tumor stage, MLH1 immunoexpression also increased. The possible explanation to this is that as the oral cancer progresses and spreads to adjacent tissues, there is an exuberant attempt to repair the defective gene, and control the unchecked proliferation of cell.

instability is a crucial early event in tumorigenesis. Promoter methylation is stated to be one of the mechanisms responsible for the loss of the MLH1 expression. Alterations such as MSI and hypermethylation of promoter regions of MLH1 and MSH2 have been detected in oral dysplasias and squamous cell carcinomas (21, 22). A few studies have found a positive correlation between loss of heterozygosity (LOH) and loss of expression for mismatch repair proteins. It is also likely that MSI develops subsequently in these cases with the tumor progression (23).

The effects of MMR proteins on the treatment aspects have been extensively studied. DNA MMR deficient cells have high mutation rates not only in the noncoding microsatellite sequences but also in genes and loci controlling drug resistance. This process makes the MLH1 deficient cells multidrug resistant, thus encompassing a significant consequence on the cancer treatment (7, 24, 25).

Our study contributes to the significant association of DNA mismatch repair gene MLH1 in oral carcinogenesis. The exact role of DNA mismatch repair genes in controlling the cell cycle proliferation, repair of the DNA defects and multidrug resistance needs to be illustrated. Further studies with wider criteria and by assessing a family of DNA repair proteins may provide useful information for assisting in diagnosis, prognostication of individual cases and redefining of therapeutic strategies.

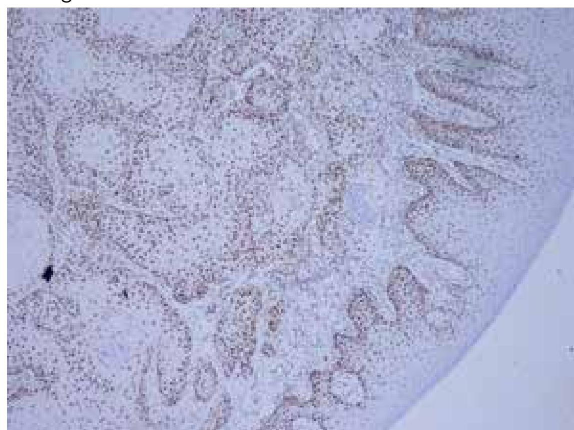

Fig 1 MLH1 staining in well-differentiated OSCC (IHC-4X)

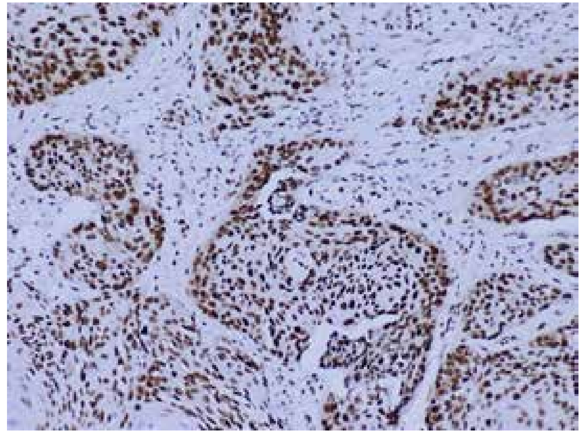

Fig 2 Intense MLH1 staining of tumor cells (IHC 20 X)

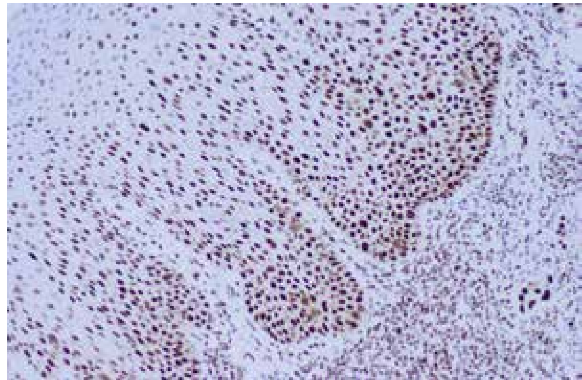

Fig 3 Intense MLH1 staining in dysplastic margins in well-differentiated OSCC (IHC 20X)

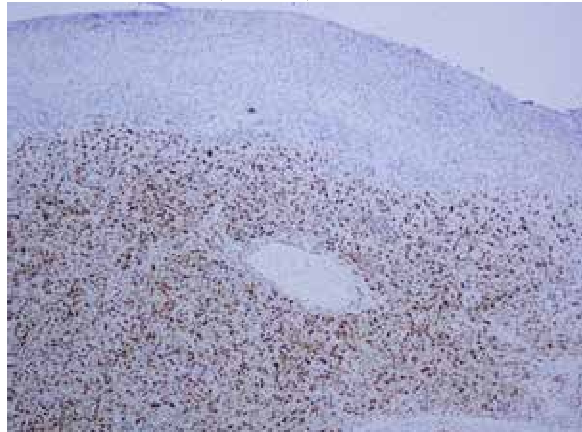

Fig 4 Loss of MLH1 staining in dysplastic epithelium in moderately differentiated OSCC (IHC 4X)

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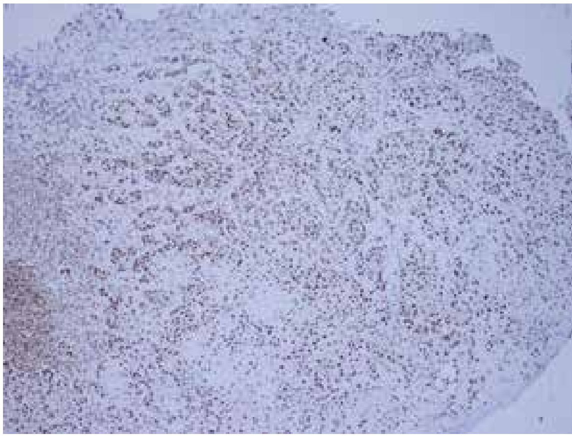


Fig 5 MLH1 staining in moderately differentiated OSCC (IHC 4X)

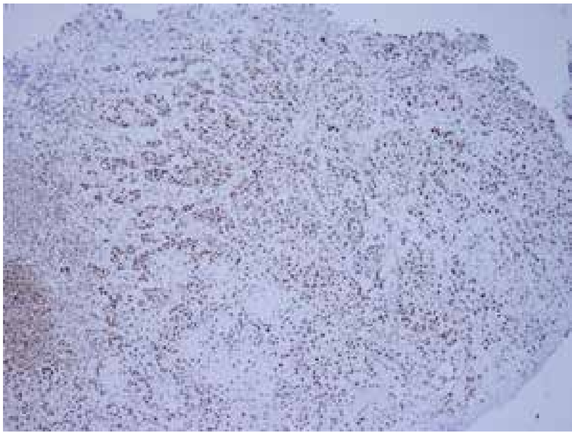
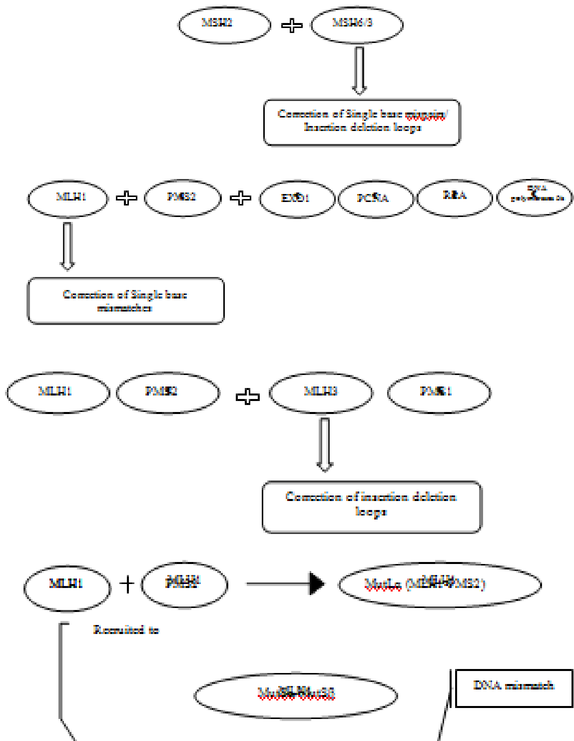
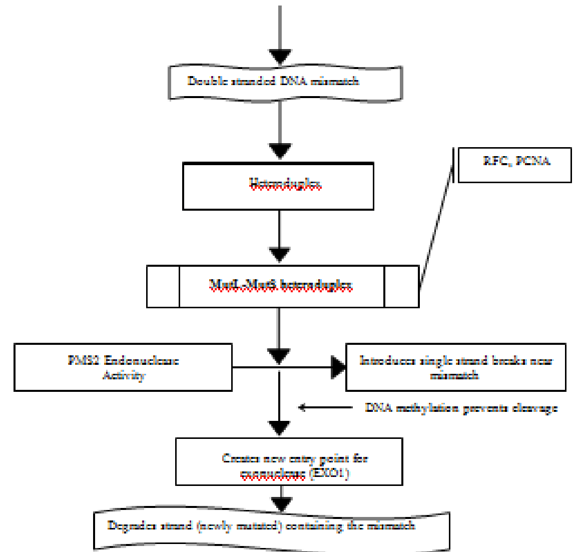


Fig 6 MLH1 staining in poorly differentiated OSCC (IHC 4X)





PMS2, **post meiotic segregation increased 2 (mismatch repair endonuclease);** *MutS*, **Mutator S;** *MSH2*, **MutS homolog 2;** *MSH6*, **MutS homolog 6;** *dsDNA*, **double stranded DNA;** *RFC*, **Replication factor C;** *PCNA*, **proliferating cell nuclear antigen**

Fig 7 Role of mismatch system in human DNA lesions

Fig 8 The mechanism associated with tumorigenesis due to defect in MLH1

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Table 1 Clinico-pathological details of oral squamous cell carcinoma patient

Case #	Age	Sex	Site	Habits	Oral hygiene	TNM stage	Grade	MLH1 score	Follow-up
1	60	M	RMT	Tobacco smoking	P	A	WD	OE	-
2	52	F	LAR	Areca nut chewing	P	A	WD	OE	Local recurrence after 1 yr
3	67	M	RMT	Smoking & Alcohol	P	E	WD	OE	-
4	62	M	BM	Tobacco & Areca nut chewing	P	A	WD	OE	-
5	49	F	T (Anterior 2/3rd)	No habits	P	E	WD	NE	-
6	60	M	LAR	Tobacco & Areca nut chewing	P	E	WD	OE	-
7	26	M	BM	Tobacco/Areca nut chewing & Alcohol	P	E	WD	OE	NER 3 yr
8	50	M	BM	Smoking & Tobacco/Areca nut chewing & alcohol	P	A	WD	OE	-
9	60	M	BM	Areca nut chewing	P	E	WD	OE	-
10	48	M	UAR	No habits	F	A	WD	OE	-
11	65	M	LAR	Smoking & Tobacco/Areca nut chewing & alcohol	P	A	WD	OE	NER 3.5 yr
12	34	F	T (Anterior 2/3rd)	Areca nut chewing	P	E	WD	OE	NER 2.8 yr
13	60	F	HP	Tobacco & Areca nut chewing	P	E	WD	OE	-
14	84	M	FOM	Tobacco/Areca nut chewing & Alcohol	P	A	WD	OE	NER 3.8 yr
15	52	F	T(Anterior 2/3rd)	No habits	F	E	WD	OE	NER 4.2 yr
16	77	M	RMT	Smoking & Tobacco/Areca nut chewing & alcohol	P	A	WD	OE	NER 3.5 yr
17	75	M	T (Anterior 2/3rd)	Areca nut chewing	P	E	WD	OE	-
18	71	M	LAR	Tobacco & Areca nut chewing	P	A	WD	OE	-
19	55	F	LAR	Areca nut chewing	P	A	WD	NE	NER 4.2 yr
20	60	M	BM	Tobacco & Areca nut chewing	P	A	WD	OE	-
21	31	M	L	Smoking & Tobacco/Areca nut chewing	P	E	WD	OE	Death 1 month post surgery
22	48	F	BM	No habits	F	A	MD	OE	NER 4 yr
23	54	M	T(Anterior 2/3rd)	Smoking & Alcohol	P	A	MD	NE	NER 4.5 yr
24	50	M	T(Anterior 2/3rd)	Smoking & Tobacco/Areca nut chewing	P	A	MD	OE	-
25	55	M	UAR	Tobacco chewing	P	A	MD	OE	-
26	65	M	BM	Tobacco smoking	P	A	MD	NE	Local recurrence after 1 yr
27	37	M	BM	Tobacco chewing	P	E	MD	RE	-
28	58	M	LAR	Tobacco & Areca nut chewing	P	A	MD	NE	-
29	30	F	BM	Tobacco chewing	P	E	MD	RE	-
30	66	F	T (Anterior 2/3rd)	Areca nut chewing	P	A	MD	NE	Local recurrence & death after 1 yr
31	42	M	BM	Tobacco & Areca nut chewing	P	A	MD	NE	NER 3.8 yr
32	45	M	UAR	Tobacco & Areca nut chewing	P	A	MD	NE	-
33	70	M	BM	Tobacco & Areca nut chewing	P	A	MD	OE	-
34	40	M	LAR	Tobacco & Areca nut chewing	P	E	MD	NE	NER 4 yr
35	52	M	LAR	Tobacco smoking	P	A	MD	NE	-
36	40	M	BM	Tobacco chewing	P	A	MD	OE	NER 4 yr
37	50	M	T (Anterior 2/3rd)	Areca nut chewing	F	A	MD	OE	NER 2.8 yr
38	65	M	BM	Areca nut chewing	P	A	MD	NE	-
39	56	M	T (Anterior 2/3rd)	Tobacco/Areca nut chewing & Alcohol	P	A	MD	NE	NER 3 yr
40	50	M	FOM	Tobacco/Areca nut chewing & Alcohol	P	A	MD	OE	-
41	56	M	LAR	Tobacco/Areca nut chewing & Alcohol	P	A	MD	NE	-
42	54	M	UAR	Tobacco chewing	P	A	PD	NE	NER 3yr
43	52	M	T (Anterior 2/3rd)	Tobacco/Areca nut chewing & Alcohol	P	E	PD	NE	NER 3.5 yr
44	60	M	T (Anterior 2/3rd)	Tobacco smoking	P	E	PD	RE	NER 3 yr
45	72	F	UAR	Tobacco & Areca nut chewing	P	A	PD	NE	NER 3.5 yr
46	52	F	LAR	Areca nut chewing	P	A	PD	RE	Local recurrence after 1 yr
47	55	M	BM	Tobacco chewing	P	A	PD	OE	-
48	64	F	BM	Areca nut chewing	P	A	PD	NE	Local recurrence & death after 3 yr
49	55	M	BM	Tobacco smoking	P	A	PD	NE	
50	70	M	LAR	Tobacco smoking	P	A	PD	OE	Local recurrence after 2 yr
51	82	M	BM	Tobacco & Areca nut chewing	P	E	PD	RE	NER 4 yr
52	65	M	BM	Areca nut chewing	P	A	PD	NE	NER 3 yr
53	44	M	T (Anterior 2/3rd)	Tobacco chewing	P	A	PD	NE	NER 4yr
54	29	M	T (Anterior 2/3rd)	Areca nut chewing	P	A	PD	NE	Death after 3 yr
55	65	F	BM	Tobacco & Areca nut chewing	P	E	PD	RE	NER 3.5 yr
56	48	F	T (Anterior 2/3rd)	Tobacco & Areca nut chewing	P	E	PD	NE	-

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57	40	F	BM	Tobacco chewing	P	A	PD	RE	NER 3 yr
58	45	M	BM	Smoking & Tobacco/Areca nut chewing	P	A	PD	OE	NER 4 yr
59	70	F	UAR	Tobacco chewing	P	A	PD	NE	NER 3.5 yr
60	55	M	T (Anterior 2/3rd)	Smoking & Alcohol	P	A	PD	NE	N NER 3 yr

M, male; F, female; BM, buccal mucosa; FOM, floor of mouth; HP, hard palate; LAR, lower alveolar ridge; UAR, upper alveolar ridge; L, lip; RMT, retromolar trigone; T, tongue; P, poor; F, fair; E, early; A, advanced; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; OE, overexpression; NE, normal expression; RE, reduced expression; NER, no evidence of recurrence; yr, year.

Table 2. Association of MLH1 expression with clinicopathological parameters in oral squamous cell carcinoma patients

	MLH1 expression			
	Reduced expression	Normal expression	Over expression	P value
n=60	7 (11.66%)	24 (40.0%)	29 (48.3%)	
Age				
< or =60 years	5 (8.3%)	17 (28.3%)	20 (33.3%)	0.985
Above 60	2 (3.3%)	7 (11.7%)	9 (15.0%)	
Gender				
Male	3 (5.0%)	17 (28.3%)	24 (40.0%)	0.094
Female	4 (6.7%)	7 (11.7%)	5 (8.3%)	
Site				
Lining mucosa	5 (8.3%)	6 (10.0%)	13 (21.7%)	0.150
Attached mucosa	1 (1.7%)	9 (15.0%)	11 (18.3%)	
Tongue	1 (1.7%)	9 (15.0%)	5 (8.3%)	
Habits				
Tobacco smoking	1 (1.7%)	3 (5.0%)	2 (3.3%)	0.681
Tobacco chewing	6 (10.0%)	15 (25.0%)	15 (25.0%)	
Smoking & smokeless tobacco	0 (0.0%)	1 (1.7%)	3 (5.0%)	
Tobacco habit & alcohol	0 (0.0%)	4 (6.7%)	6 (10.0%)	
No habits	0 (0.0%)	1 (1.7%)	3 (5.0%)	
Oral hygiene				
Fair	0 (0.0%)	0 (0.0%)	4 (6.7%)	0.101
Poor	7 (11.7%)	24 (40.0%)	25 (41.7%)	
Staging				
Early stage	5 (8.3%)	4 (6.7%)	9 (15.0%)	0.021*
Advanced stage	2 (3.3%)	20 (33.3%)	20 (33.3%)	
Histologic grading				
Well differentiated	0 (0.0%)	2 (3.3%)	19 (31.7%)	0.000*
Moderately differentiated	2 (3.3%)	11 (18.3%)	7 (11.7%)	
Poorly differentiated	5 (8.3%)	11 (18.3%)	3 (5.0%)	

*(P value < 0.05 considered statistically significant)

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