Original Article

Human MutL homolog 1 immunoexpression in oral leukoplakia and oral squamous cell carcinoma: A prospective study in Indian population

Narendra T Chaudhari, Jagdish V Tupkari, Tabita Joy, Manisha S Ahire

Department of Oral Pathology and Microbiology, Government Dental College and Hospital, Mumbai, Maharashtra, India

Abstract

Background: Mammalian mismatch repair system is responsible for maintaining genomic stability during repeated duplications, and human MutL homolog 1 (hMLH1) protein constitutes an important part of it. Various isolated studies have reported the altered expression of hMLH1 in oral leukoplakia (OL) and oral squamous cell carcinoma (OSCC). Research is lacking in the quantitative estimation and comparison of hMLH1 expression in OL and OSCC.

Aims: To evaluate, quantify and compare hMLH1 immunoexpression in normal oral mucosa, OL and OSCC.

Settings and Design: Thirty patients of OL and thirty patients of OSCC formed the study group and thirty patients were included in the control group (normal oral mucosa). Formalin-fixed paraffin wax blocks were prepared from the tissue samples.

Materials and Methods: Immunohistochemistry for hMLH1 was performed, and the total number of positive cells was counted in high-power fields, and based on that percentage positivity of hMLH1 was calculated in all the cases.

Statistical Analysis: Kruskal–Wallis and t-test were used. $P < 0.05$ was considered to be statistically significant.

Results: The mean hMLH1 value in control group, leukoplakia and OSCC was 78.26, 54.33 and 40.97 respectively. hMLH1 immunoexpression showed decreasing indexes from control group to leukoplakia and then further to OSCC. hMLH1 expression was significantly lower in OSCC as compared to leukoplakia. There was no significant correlation of mean hMLH1 expression between different clinical and histopathological stages of leukoplakia and OSCC.

Conclusions: hMLH1 immunoexpression was inversely related to the degree of dysplasia. These findings suggest that there is a progressive decrease in hMLH1 expression from control to leukoplakia and further to OSCC. Thus, it can be concluded that hMLH1 can be used as a reliable biomarker for malignant transformation.

Key Words: Mismatch repair system, oral leukoplakia, oral squamous cell carcinoma

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common type of head and neck cancer,[1] with an annual incidence of 350,000 cases worldwide.[2] Despite several treatment modalities in the last three decades, it has 50% survival rate over 5 years because of late diagnosis. It is the 11th most common cancer worldwide and 8th most frequent cancer in the world in males and 14th in females. OSCC accounts for nearly 3% of all cancer cases.[1,3]

OSCC can be preceded by oral leukoplakia (OL), the main oral potentially malignant disorder.[4,5] The frequency of dysplasia varies from 15.6 to 39.2% in OL. Although the histological investigation is the routinely used method for grading epithelial dysplasia, it is associated with interobserver variability.[6] To overcome this, and further identify the early events involved in OL to OSCC cellular transformation, research in biological markers is necessary.[7]

Immunohistochemistry is quick and cheap as compared to genetic analysis in proving the loss of protein expression.[8] One of the recently evolving biomarkers is the human MutL homolog 1 (hMLH1) immunohistochemical stain.[9]

The mammalian mismatch repair (MMR) system is responsible for maintaining genomic stability during repeated duplication.[10] The hMLH1 forms an important part of MMR and plays a major role in mutation avoidance.[11] Mutation of hMLH1 gene is seen in hereditary nonpolyposis colorectal carcinoma[12] which may occur due to MMR gene mutation or promoter methylation gene silencing.[13] Microsatellite instability (MSI) may result in genomic instability[14,15] and serve as a crucial early event in carcinogenesis.[16] Alterations such as MSI and hypermethylation of the promoter regions of hMLH1 were detected in OL and OSCC.[17]

However, very few studies have been done in relation to oral potentially malignant disorders and oral cancer. Hence, the aim of the present study was to compare the immunoexpression of hMLH1 in different stages of OL and OSCC with that of normal healthy mucosa, and to determine whether hMLH1 is a reliable biomarker for malignant transformation.

MATERIALS AND METHODS

Study group
Inclusion criteria
- Clinically suspicious and diagnosed cases of OL and OSCC
- Individuals should be otherwise healthy, with no bar for age and sex
- Individuals willing to participate in the study procedure with written consent.

Exclusion criteria
- Individuals having any systemic illness such as diabetes and hypertension.
- Individuals having oral ulcerative lesions (traumatic ulcers, herpetic lesions, etc.) which are not clinically suggestive of cancer
- Individuals having white lesions not associated with tobacco use
- Individuals unwilling to participate in the study.

Control group
Inclusion criteria
- Individuals should be otherwise healthy, with no bar for age and sex
- Individuals with no habit history (tobacco).

Exclusion criteria
- Individuals having any systemic illness such as diabetes and hypertension.
- Individuals unwilling to participate in the study.

Tissue samples
The study protocol was approved by the Ethics Committee of our hospital. Thirty randomly selected patients of OL were clinically grouped using LCP classification (L - size, C - clinical presentation and P – pathological grading) (Bailoor and Nagesh, 2005).[18] H and E stained sections were analyzed by two investigators and the degree of epithelial dysplasia was established in accordance with the criteria given by WHO.[19]

Thirty randomly selected patients of OSCC were clinically grouped using tumor node metastasis classification.[20] Slides were examined and diagnosed histopathologically as OSCC and scored as per the criterion given by Anneroth and Hansen[21] with modification like the "the stage of invasion". The depth of tumor cell infiltration was excluded as the majority of specimens were obtained from incisional biopsy. The scores and prognosis were determined from the total malignancy score [Table 1]. Thirty patients were included in control group that comprised healthy volunteers (normal oral mucosa obtained from crown lengthening procedure and third molar surgery).

Immunohistochemical staining
Three to four micrometers thick formalin-fixed paraffin-embedded sections were dewaxed in xylene and hydrated with graded ethanol. Antigen retrieval was done in a microwave by placing slides in a bath containing 250 ml of

Table 1: Correlation of malignancy score, grade and prognosis

Malignancy score	Grade	Prognosis
5-8	I	Good
9-12	II	Moderate
13-20	III	Poor

working strength antigen retrieval citra plus solution, turning heat to high and then incubating for 10–15 min. Slides were transferred to room temperature and washed with ethylene-di-amine-tetra-acetic acid solution with a pH of 9.0.

Endogenous peroxidase was blocked with a 1:1 solution of methanol and 3% H_2O_2 for 10 min. The slides were incubated with prediluted primary antibody anti-hMLH1 (monoclonal mouse) (DAKO Corporation, Glostrup, Denmark, Clone ES05). Detection was undertaken with a two-step, highly sensitive, ready-to-use, peroxidase-based system named visualization system (DAKO Corporation, Denmark). Reactions were revealed with 3,3'-diaminobenzidine chromogen solution. Harri's hematoxylin was used for counterstaining. Negative controls were obtained by the omission of primary antibody and samples of normal oral mucosa with known positive reactivity were included as positive controls.

Cell counting and statistical analysis

Cells were considered immunopositive, if they presented brown nuclear staining, regardless of intensity. Cell counting was performed using an eyepiece grid in light microscopy under ×400. Counting was done in suprabasal cell layer in cases of normal oral mucosa and leukoplakia, whereas in case of OSCC, all tumor cells were counted in a particular field.

According to the analysis performed by Fernandes *et al.* to obtain hMLH1 index, 16 high-power fields (×400) were analyzed for each slide and positive and negative cells were counted. The number of positive cells was divided by the total number of cells counted in all the fields, i.e. positive and negative ones and the result was then multiplied by 100, so the indexes were demonstrated as percentage of positive cells.[9]

Statistical analysis was performed using SPSS software version 12.0. (SPSS Inc., 233 South Wacker Drive, 11th floor, Chicago, IL 60606-6412). Kruskal–Wallis test was applied to compare mean hMLH1 expression in control, leukoplakia and OSCC groups. *T*-test for equality of means (2-tailed) was used to compare the clinical and histopathological stages of both leukoplakia and OSCC with that of control group. The results were considered statistically significant with $P < 0.05$.

RESULTS

The demographics and site distribution of the present study are as presented in Table 2.

Human MutL homolog 1 immunoexpression

hMLH1 positivity was seen in all the ten samples of normal oral mucosa [Figure 1]; 25 of 30 samples of leukoplakia [Figures 2 and 3] and 24 of 30 samples of OSCC [Figures 4-6]. Most of the patients of the study group had similar habit history, age group and site of lesion. In addition, there were no female patients in leukoplakia group, hence these parameters were considered nonsignificant and were not compared.

The mean value of hMLH1 expression in normal, leukoplakia and OSCC patients was calculated. hMLH1 expression was significantly higher in control group than both leukoplakia and OSCC groups. Between leukoplakia and OSCC groups, hMLH1 expression was significantly higher in leukoplakia as $P < 0.05$ (Kruskal–Wallis test) [Table 3].

Mean hMLH1 expression was significantly higher in control group as compared to stages 1, 2, 3 and 4 of leukoplakia, as $P < 0.05$ (*t*-test). There was no significant difference of hMLH1 expression between the different clinical stages of leukoplakia, as $P > 0.05$ (*t*-test) [Table 4].

Mean hMLH1 expression was significantly higher in control group as compared to mild as well as moderate

Table 2: The demographics and site distribution of the present study

| Patients and clinical details	Oral Leukoplakia (*n*=30)	OSCC (*n*=30)
Age range (years)	23–60	32–70
Male/female	30/0	19/11
Buccal mucosa	22	15
Alveolar ridge	–	6
Buccal vestibule	3	5
Labial mucosa	2	1
Tongue	–	2
Retromolar area	1	–
Gingiva	1	1
Palate	1	–

OSCC: Oral squamous cell carcinoma
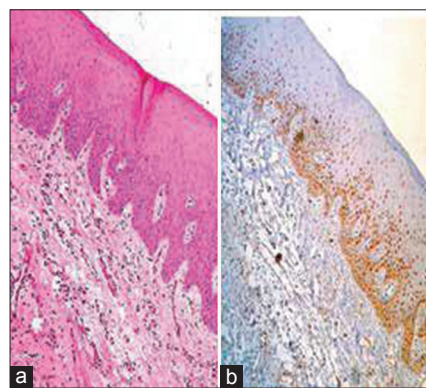
Figure 1: (a) Photomicrograph showing parakeratinized stratified squamous epithelium (control) (H&E stain, ×100). (b) Positive human MutL homolog 1 expression predominantly in basal and suprabasal layer of normal mucosa (control) (IHC stain, ×100)
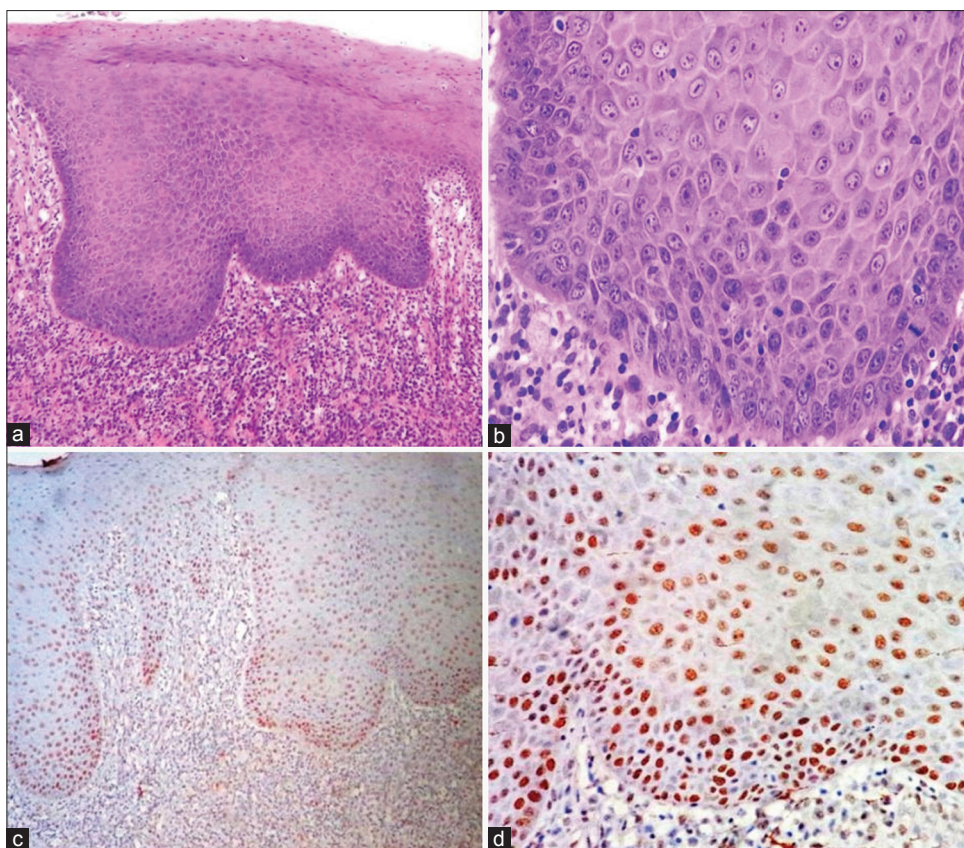
Figure 2: (a) Oral Leukoplakia; Photomicrograph showing mild dysplasia (H&E stain, ×100). (b) Oral Leukoplakia; Photomicrograph showing mild dysplasia (H&E stain, ×400). (c) Oral Leukoplakia; positive human MutL homolog 1 staining in mild dysplasia (IHC stain, ×100). (d) Oral Leukoplakia; positive human MutL homolog 1 staining in mild dysplasia (IHC stain, ×400)
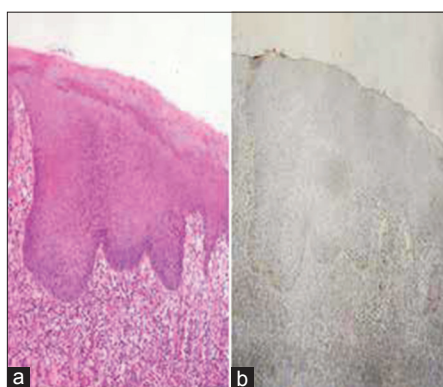
Figure 3: (a) Oral Leukoplakia; Photomicrograph showing moderate dysplasia (H&E stain, ×100). (b) Oral Leukoplakia; positive human MutL homolog 1 staining in moderate dysplasia (IHC stain, ×100)

Table 3: Mean value of human MutL homolog 1 expression in normal, oral leukoplakia and oral squamous cell carcinoma patients

| Group	Mean hMLH1 percent score	n	Mean rank	χ^2	df	P
Control	78.26	30	65.5	37.984	2	0.000
Oral Leukoplakia	54.33	30	39.78			
Oral Squamous cell carcinoma	40.97	30	21.22			
Total		70				

hMLH1: Human MutL homolog 1

dysplasia in leukoplakia, as $P < 0.05$ (*t*-test). There was no significant difference of hMLH1 expression between the different histopathological grades of leukoplakia, as $P > 0.05$ (*t*-test) [Table 5].

Mean hMLH1 expression was significantly higher in control group as compared to stages I, II, III and IV of OSCC, as $P < 0.05$ (*t*-test). There was no significant difference of hMLH1 expression between the different clinical stages of OSCC, as $P > 0.05$ (*t*-test) [Table 6].

Mean hMLH1 expression was significantly higher in control group as compared to Grades I, II and III of OSCC, as $P < 0.05$ (*t*-test). There was no significant difference of hMLH1 expression between the different histopathological grades of OSCC, as $P > 0.05$ (*t*-test) [Table 7].

The association of tobacco use was assessed neither for OL nor for OSCC, as all the patients were tobacco users
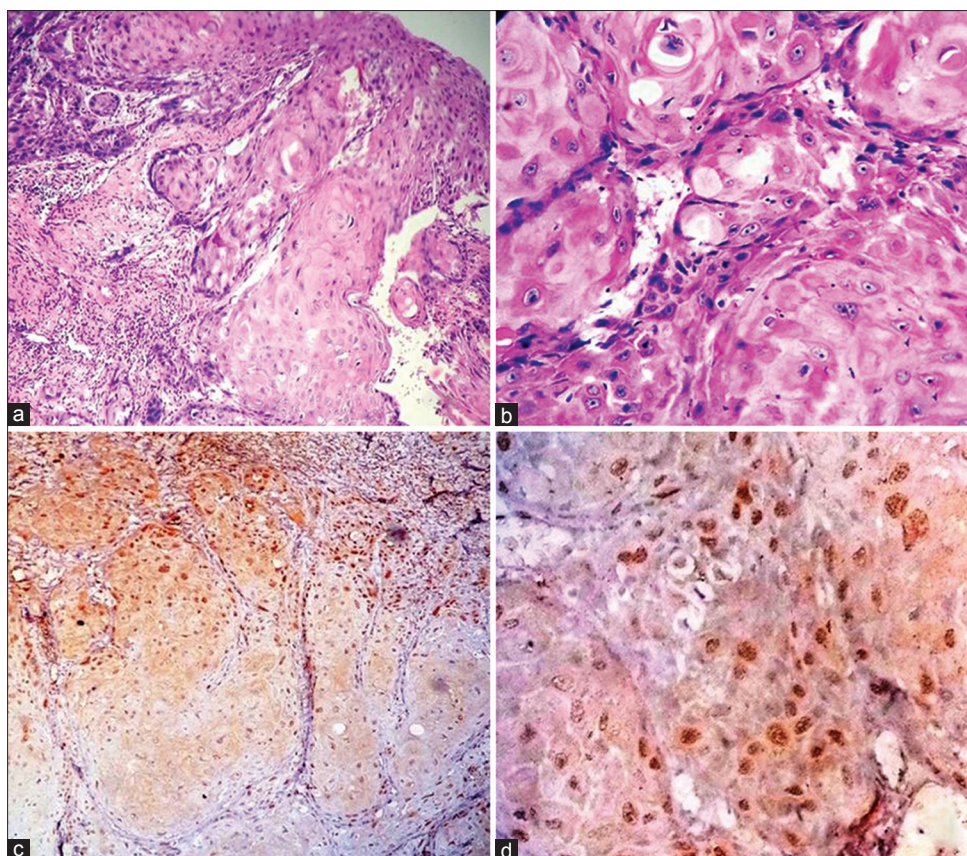
Figure 4: (a) Photomicrograph of oral squamous cell carcinoma Grade I (H&E stain, ×100). (b) Photomicrograph of oral squamous cell carcinoma Grade I (H&E stain, ×400). (c) Positive human MutL homolog 1 staining in oral squamous cell carcinoma Grade I (IHC stain, ×100). (d) Positive human MutL homolog 1 staining in oral squamous cell carcinoma Grade I (IHC stain, ×400)

in some or the other forms with variable duration and frequency.

DISCUSSION

Oral carcinogenesis is a multistep process, in which epigenetic changes form an important mechanism in oral cancer development, and their recognition may help in early detection as well as development of new therapeutic strategies. The DNA MMR pathway corrects replicate mismatches that escape DNA polymerase proofreading, and hence plays an important role in the maintenance of genetic stability.[22]

DNA repair forms an important defense mechanism against DNA damage and alteration of MMR proteins such as Mut-L-Homologon-1 (MLH1) that have recently been implicated in the development, progression and metastasis of several types of head and neck neoplasias. MLH1 forms heterodimers with PMS2 and MLH3 (MutL complex) to discriminate the old from the new DNA strand and to signal downstream repair factors such as helicases and exonucleases. Tobacco-addicted patients with head and neck cancer are more susceptible to gene inactivation of hMLH1 genes by promoter

Table 4: Mean human MutL homolog 1 expression between control and clinical stages of oral leukoplakia

Clinical LCP stage	Number of cases	Percentage hMLH1 positivity (mean)
Stage I	9	56.54
Stage II	4	61.79
Stage III	1	61.58
Stage IV	16	50.76

T-test for equality of means, *P* value (two-tailed)				
Group	Control	Stage IV	Stage III	Stage II	Stage I
Stage I	0.006	0.623	0.833	0.658	-
Stage II	0.000	0.491	0.9823	-	0.658
Stage III	0.000	0.735	-	0.983	0.833
Stage IV	0.009	-	0.735	0.491	0.623

hMLH1: Human MutL homolog 1, LCP: L - size, C: Clinical presentation, P: Pathological

hypermethylation.[23] Hypermethylation of hMLH1 was found in 0–47% of HNSCC[22] and 14–70% of leukoplakia with a higher prevalence of MSI in leukoplakia showing severe degrees of dysplasia.[9]

In this study, no relationship was found between hMLH1 immunoexpression and gender, age or sample site between control, leukoplakia and OSCC groups. This result may well
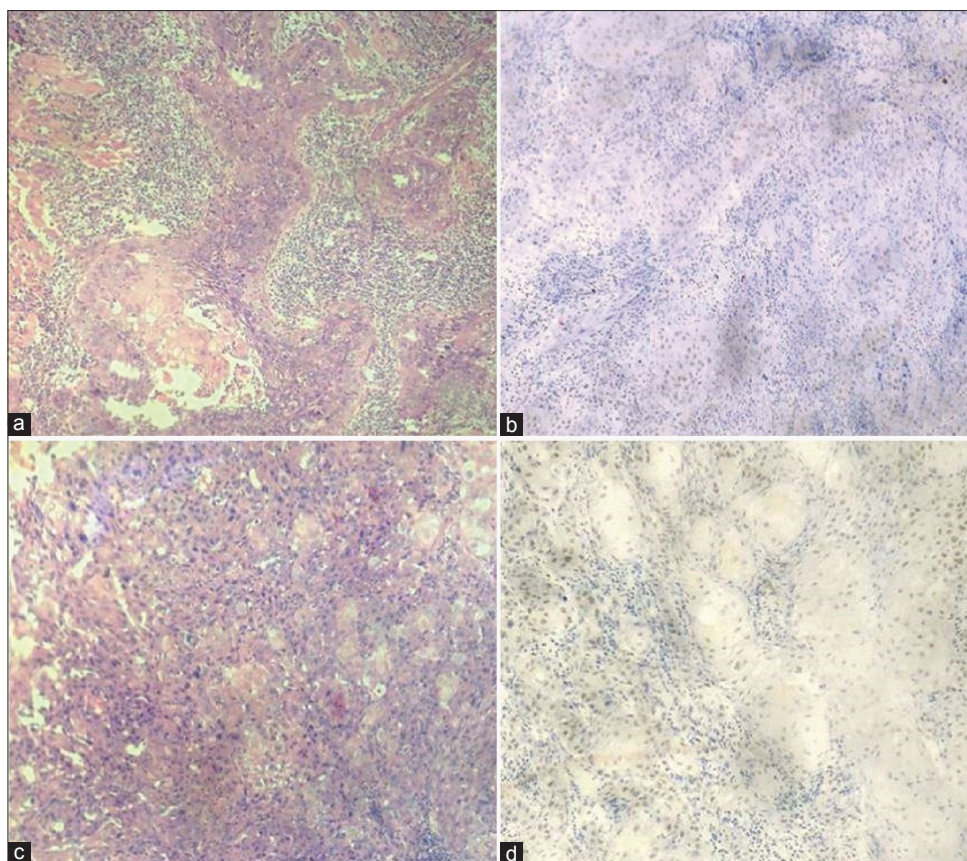
Figure 5: (a) Photomicrograph of oral squamous cell carcinoma Grade II (H&E stain, ×100). (b) Faint human MutL homolog 1 staining in oral squamous cell carcinoma Grade II (IHC stain, ×100). (c) Photomicrograph of oral squamous cell carcinoma Grade III (H&E stain, ×100). (d) Faint human MutL homolog 1 staining in oral squamous cell carcinoma Grade III (IHC stain, ×100)

Table 5: Comparison of mean human MutL homolog 1 expression between control and histopathological grades of oral leukoplakia

Histopathological grade	Number of cases	Percentage hMLH1 positivity (mean)
Mild dysplasia	25	57.44
Moderate dysplasia	5	38.75

| *T*-test for equality of means, *P* value (two-tailed)			
Group	Control	Moderate dysplasia	Mild dysplasia
Mild dysplasia	0.006	0.133	-
Moderate dysplasia	0.003	-	0.133

hMLH1: Human MutL homolog 1

reflect the basic and primitive function of the MMR system which is conserved throughout evolution and unaltered by demographic variations.[24] Immunohistochemical staining procedure was repeated for the negative cases to rule out technical errors owing to the sensitivity of the procedure. However, repeated staining also showed similar results and therefore these 11 cases (5 of leukoplakia and 6 of OSCC) were considered negative for hMLH1. This could be due to promoter hypermethylation of the hMLH1 gene owing to carcinogens from tobacco such as oxygen-based free radicals, peroxides and peroxinitrite, which cause severe oxidative stress. Reactive oxygen species can directly oxidize DNA, resulting

in mutagenic change and may damage some DNA repair proteins.[24] In addition, the antigen levels may be too low for detection by the employed staining method. Loss of antigenic differentiation in some tumors or loss of antigenicity due to suboptimal or excessive tissue fixation may result in negative expression. Immunoreactivity is diminished or destroyed when paraffin used for embedding process exceeds 60°C.[25]

The present study had few observations similar to Fernandes *et al.*,[8] which are as follows:

- The study and control groups had a wide range of hMLH1 expression, which may be due to different transcriptional and translational control of hMLH1 gene[24]
- In this study, some cases showed hMLH1 immunoexpression in cytoplasm which may be due to action of the MMR system in mitochondrial DNA, similar to that which occurs in the nucleus,[8] so, only those cases showing distinct nuclear immunoreactivity were considered positive for hMLH1
- The cells of minor salivary glands, the nucleus of muscle cells and mononuclear leukocytes (when present in stroma) showed nuclear staining, its significance is yet unknown[8]
- The staining pattern observed in our study was heterogeneous, i.e. all the cells in the positive cases did not express hMLH1
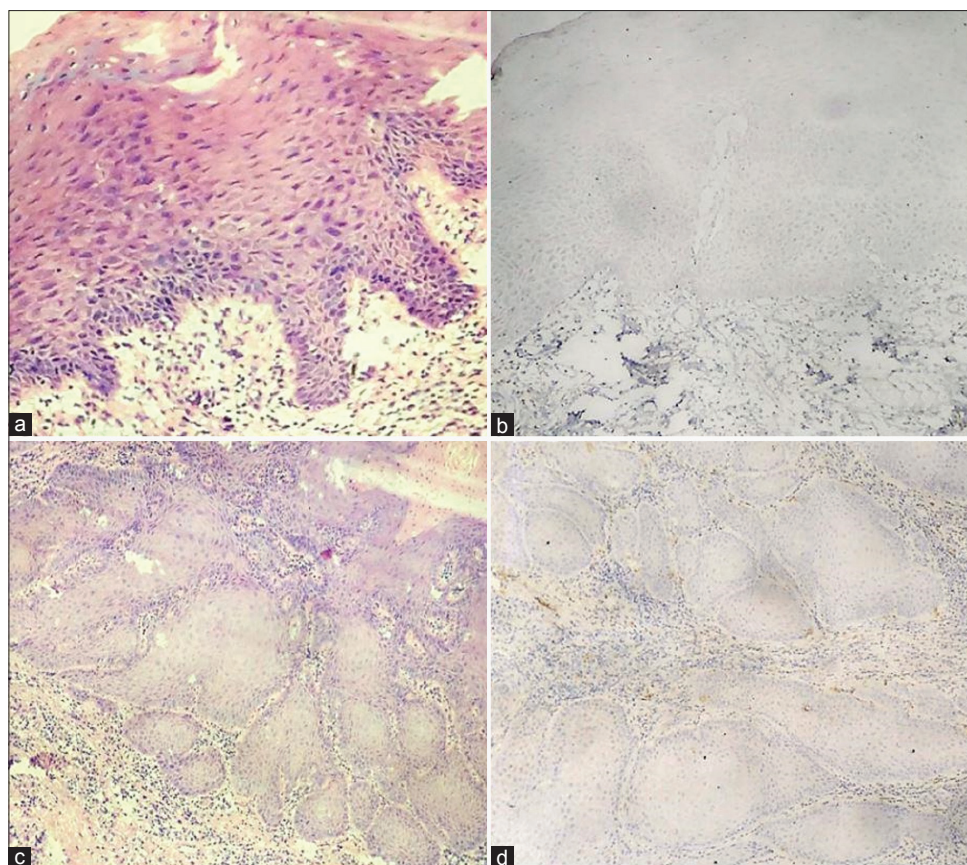
Figure 6: (a) Photomicrograph of oral leukoplakia (H&E stain, ×100). (b) Negative human MutL homolog 1 staining in oral leukoplakia (IHC stain, ×100). (c) Photomicrograph of oral squamous cell carcinoma (H&E stain, ×100). (d) Negative human MutL homolog 1 staining in oral squamous cell carcinoma (IHC stain, ×100)

Table 6: Comparison of mean human MutL homolog 1 expression between control and clinical stages of oral squamous cell carcinoma

Clinical TNM stage	Number of cases	Percentage hMLH1 positivity (mean)
1	2	24.62
2	4	19
3	23	45.54
4	1	48.52

T-test for equality of means, *P* value (two-tailed)					
Group	Control	Stage 4	Stage 3	Stage 2	Stage 1
Stage 1	0.000	0.675	0.175	0.965	-
Stage 2	0.000	0.468	0.057	-	0.965
Stage 3	0.000	0.882	-	0.057	0.175
Stage 4	0.000	-	0.882	0.468	0.695

hMLH1: Human MutL homolog 1, TNM: Tumor node metastasis

which may be due to a different frequency of heterozygosity loss and MSI in different areas of leukoplakia and OSCC because of intratumor genetic heterogeneity.[8]

Mean value of human MutL homolog 1 expression in control, oral leukoplakia and oral squamous cell carcinoma patients

There was a progressive decrease in hMLH1 expression from control to leukoplakia and further to OSCC [Table 3],

which may be due to the presence of MSI and epigenetic alterations in leukoplakia as well as occurrence of hMLH1 gene hypermethylation, leading to a reduced immunoexpression of this protein in squamous cell carcinoma of the head and neck. Caldiera *et al*. (2011) noted that hMLH1 immunoexpression showed decreasing indexes from lesions with lower degrees of dysplasia to lesions with more severe dysplasia. de Oliveira *et al*. (2014) reported that a higher percentage of epithelial cells expressed hMLH1 in cases of actinic cheilitis without dysplasia or mild dysplasia as compared to lower lip squamous cell carcinomas. Tobacco use would lead to epigenetic alterations of MMR genes in normal oral mucosa, which would then become more susceptible to malignant transformation, possibly with MSI phenotype.[26,27] The reduced expression may be because of exhaustion of MMR system owing to constant carcinogenic exposure.[8]

Mean value of human MutL homolog 1 expression in clinical stages of oral leukoplakia and control groups

Mean hMLH1 expression was significantly higher in control group as compared to different clinical stages of leukoplakia [Table 4]. This shows that MMR system is affected as the severity of leukoplakia increases clinically when compared

Table 7: Comparison of mean human MutL homolog 1 expression between control and histopathological grades of oral squamous cell carcinoma

Histopathological grade	Number of cases	Percentage hMLH1 positivity (mean)
I	2	51.93
II	23	38.29
III	5	50.89
T-test for equality of means, *P* value (two-tailed)		
Group	Control	III	II	I
I	0.000	0.899	0.438	-
II	0.000	0.265	-	0.438
III	0.000	-	0.265	0.899

hMLH1: Human MutL homolog 1

with that of normal mucosa. There was no significant difference of hMLH1 expression between the different clinical stages of leukoplakia. This may be due to the fact that leukoplakia may present different clinical behavior and biological evolution, as the predictors of malignant transformation depend on several factors such as the duration of the lesion, patient's age and gender, the affected site, clinical appearance, smoking habit and presence of epithelial dysplasia.[9]

Mean value of human MutL homolog 1 expression in histopathologic grades of oral leukoplakia and control groups

Mean hMLH1 expression was significantly higher in control group as compared to mild as well as moderate dysplasia in leukoplakia group [Table 5]. These findings suggest that there is an alteration in DNA repair pathway, particularly in hMLH1 gene, with an increase in the severity of dysplasia of leukoplakia when compared to normal mucosa.[26] There was a difference of mean hMLH1 expression between the different histopathological grades of leukoplakia, but it was not statistically significant. This could be due to subjective and lack of inter- and intra-observer reproducibility in the grading of dysplasia.[28] Leukoplakia with similar histological phenotypes may show different biological behavior.[26]

Mean value of human MutL homolog 1 expression in the clinical stages of oral squamous cell carcinoma and control groups

Mean hMLH1 expression was significantly higher in control group as compared to different stages of OSCC [Table 6]. These results suggest that hMLH1 activity reduces as the severity of OSCC increases clinically when compared with normal mucosa. There was no significant difference of hMLH1 expression among the different clinical stages of OSCC which may be due to the lack of accurate and reliable stratification of head and neck cancers because of the numerous anatomic sites and subsites from which tumors can arise and the diversity of histologic types of tumors in these locations.[29] Furthermore, all the parameters were not assessed for the clinical staging due to practical difficulties such as lack of magnetic resonance imaging scan.

Mean value of human MutL homolog 1 expression in histopathologic grades of oral squamous cell carcinoma and control groups

Mean hMLH1 expression was significantly higher in control group as compared to various grades of OSCC [Table 7]. Fernandes *et al.* reported an overexpression of hMLH1 in well-differentiated OSCCs as compared to poorly differentiated OSCCs which showed reduced hMLH1 expression. The reduced expression of hMLH1 in poorly differentiated OSCCs suggests saturation of the MMR system. On the other hand, the protein overexpression may reflect an attempt on the part of the MMR system to correct the multiplicity of mismatches.[8] There was no significant difference in hMLH1 expression among the different histopathological grades of OSCC. This could be explained by the fact that the validity of histopathologic grading as a marker of prognosis remains controversial due to tumor heterogeneity, interobserver disagreement and variations in the size of the high-power field in different microscopes.[30] In spite of our efforts, uniform sample size could not be achieved in different stages which affected statistical analysis.

CONCLUSIONS

Identification of early molecular markers that precede phenotypic alterations will help in the prediction of cancer development. Hence, despite its possible role in the development and progress of dysplastic phenotype, hMLH1 alone is not sufficient to grade epithelial dysplasia as an ample range of hMLH1 values were seen in the present study within the same group for OL and OSCC.

In the present study, significantly, lower hMLH1 expression is seen in leukoplakia, which further decreases in OSCC as compared to normal oral mucosa. Moreover, significantly reduced hMLH1 expression is seen in different clinical and histopathological stages of both leukoplakia and OSCC with respect to normal oral mucosa. Therefore, altered expression of hMLH1 in leukoplakia seems to be an early event in carcinogenesis. In addition, reduced hMLH1 expression in OSCC may reflect the saturation of DNA repair pathway and highlight the role of hMLH1 in the progression of carcinogenesis and can be considered a useful marker of poor prognosis.

Molecular research studies on MMR system might be helpful in understanding the precise mechanism of hMLH1 in potentially malignant disorders and cancer.

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Conflicts of interest
There are no conflicts of interest.

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