RESEARCH ARTICLE

Editorial Process: Submission:06/25/2024 Acceptance:05/14/2025

Mismatch Repair Proteins Immunostaining in Lip Squamous Cell Carcinoma: A Role in Lip Carcinogenesis?

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Abstract

Objective: Lip squamous cell carcinoma (LSCC) is associated with malignant transformation of actinic cheilitis (AC). Since solar radiation alters the functions of mismatch repair (MMR) complex, we evaluated for their possible role in lip carcinogenesis. **Materials and methods:** Samples of normal lip epithelia (NLE) (n=15), AC (n=30), and LSCC (n=45) were subjected to immunohistochemistry for MutSα (MSH2/MSH6) and MutLα (MLH1/PMS2) to assess the percentage (brown nuclei over all the keratinocytes in NLE and AC or all tumoral cells in LSCC) of nuclear positive cells and MSH2/MSH6 (MutSα-imbalance) and MLH1/PMS2 (MutLα-imbalance) ratios. Clinical-prognostic variables of the primary tumor and histopathological gradation (LSCC and AC) were evaluated. Mann–Whitney, Kruskal–Wallis/Dunn, and Spearman correlation tests were used ($p<0.05$, SPSS 20.0). **Results:** LSCC and AC showed significant increases in MSH2 ($p<0.001$), MSH6 ($p<0.001$), MLH1 ($p=0.040$) percentage of immunostained cells, and MutSα-imbalance ($p<0.001$). MutSα-imbalance in AC was higher than MutLα-imbalance ($p=0.028$). In LSCC, T3/T4 tumors showed higher MutSα-imbalance ($p=0.028$) and MutLα-imbalance ($p=0.014$). In LSCC with nodal metastasis, the MutLα-imbalance was significantly higher than the MutSα-imbalance ($p=0.046$). AC with high-risk dysplasia ($p=0.024$) and LSCC with vascular invasion ($p=0.035$) showed lower immunostaining for MSH6. Direct correlations between MMR-proteins increased in LSCC. **Conclusions:** Increased MMR expression in lip cancer and the imbalance between MutSa and MutLα is associated with the progression and prognosis of LSCC.

Keywords: Lip Neoplasms- MutS Proteins- MutL Proteins

Asian Pac J Cancer Prev, **26 (5)**, 1553-1562

Introduction

Lip squamous cell carcinoma (LSCC) occurs predominantly due malignant transformation of an Actinic cheilitis (AC) [1]. This carcinogenesis is related ultraviolet (UV) radiation [2, 4, 5] which is believed to be the main cause of interruption of the cell cycle and damage to DNA [6].

The biological consequences of DNA damage hinge on the injury's nature, often affecting DNA replication fidelity and causing mutations. UV radiation is widely acknowledged to induce specific signatures leading to mutations, fostering the development of squamous cell carcinomas (SCCs) and basal cell carcinoma [7]. Mismatch repair (MMR) DNA proteins play a vital role in various cellular functions. Their primary function involves correcting bases erroneously incorporated into the genome due to replication errors [3, 8].

The recognition of the incompatible MMR system is carried out by heterodimers of MSH proteins (MutS homolog). The MSH2/MSH6 (MutSα) heterodimer recognizes base incompatibilities. In contrast, the heterodimer MSH2/MSH3 (MutSβ) recognizes nucleotide deletion and incompatibility mediated by MutSα after initial recognition, which binds to the damaged DNA strand and recruits MutLα (MLH1/PMS2) [3, 9-11].

Deficiencies in Mismatch Repair (MMR) proteins have been observed in studies focusing on potentially malignant lesions, particularly in Actinic Cheilitis (AC). Immunohistochemical expression of MMR proteins in AC [12-14] revealed lower MLH1 and MSH2 expression associated with increased dysplasia [3], although higher

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MLH1 expression in AC with mild dysplasia has also been noted [12].

Recent studies highlight the correlation between immunostaining of MMR complex proteins and oral and oropharyngeal carcinogenesis, as well as the prognosis of head and neck SCCs [1, 15, 16]. This study aims to evaluate MMR complex immunostaining in lip carcinogenesis, assessing its influence on clinical and pathological variables of LSCC. The hypothesis suggests an association between MMR complex proteins and LSCC carcinogenesis.

Materials and Methods

Ethical considerations

This research was approved by the Research Ethics Committee of the Haroldo Juaçaba Hospital/Ceará Cancer Institute, under registration protocol number 2.191.839. The study is in compliance with the norms that regulate research in human beings, as stated in resolution 466/12 of the National Health Council. Furthermore, the study was conducted using the scientific methodology known as the STROBE initiative.

Sample calculation and study groups

To address the similarity between lip cancer and SCC, a sample size calculation was performed based on a prior study of head and neck SCC [17]. For the sample size, a ratio of three cases for each control (45 LSCC and 15 lip epithelia without microscopic alterations) was adopted to achieve a sample representing the hypothesis of this research with 80% power and 95% confidence. For inclusion of AC samples, a proportion of two cases for each control was set. Therefore, the total sample size for each tissue type was determined as 45 lip SCC, 30 AC, and 15 normal epithelia of the vermilion of the lower lip. The samples of LSCC, AC and NLE were collected from different patients and paraffin blocks.

Data collection

The current study collected samples from patients at Hospital Haroldo Juaçaba/Instituto do Câncer do Ceará (HHJ/ICC) who underwent surgery to resect carcinoma of the vermilion of the lips, without neoadjuvant treatment. Additional samples were obtained from NLE from oral mucoceles and squamous cell carcinoma (SCC) cases.

Clinicopathological data were collected, and suitable paraffin blocks were selected for histopathological analysis. The study excluded cases with incomplete records and blocks without sufficient material for the tissue microarray (TMA) technique.

Histopathological grading of LSCC and actinic cheilitis

The classification of ACs and lip SCCs was performed by an experienced pathologist (>10 years of oral pathology), with an intra-examiner calibration coefficient kappa = 0.859. The ACs had their epithelial dysplasia classified using a binary system of low/high risk of malignant transformation [18]. The LSCC samples were classified using Bryne's binary model, which classifies SCC into high and low grade [19]. The presence of perineural and vascular invasion was also assessed. Perineural invasion was considered when there was a presence of cancer cells within the epineurial, perineurial, and/or endoneurial compartments of a nerve in stroma (peritumor) independent regardless of the size of the nerve fiber [20]. Vascular invasion was considered when there lymphatic or blood vessels showed the presence of aggregates of tumor cells within endothelial lined spaces with no underlying muscular walls and invasion of the media of a vessel with ulceration of the intima, respectively, in stroma (peritumor) [21].

TMA and immunohistochemistry

The study involved examining microscopic slides of lip squamous cell carcinoma (LSCC) and actinic cheilitis (AC) in comparison to control regions. For the tissue microarray technique, two cores of 2mm diameter circumferential areas (3.14 mm^2) were selected from each sample using a tissue microarrayer. The samples of tumor were selected from invasive front of tumor. Equally, two cores of NLE and AC were selected in health areas of lips and dysplastic areas of AC.

So, paraffin blocks from these areas were punched and transferred to a receiver block with 70 circular wells. The resulting paraffin receptor blocks, containing oral ulcers, were then sectioned into 3-µm thick slices and placed on silanized slides for subsequent analysis.

For immunohistochemical processing, samples were deparaffinized, rehydrated, and subjected to antigen retrieval in Tris-EDTA buffer (pH 9.0). To inactivate endogenous peroxidases, samples were incubated in phosphate-buffered saline (PBS) with 3% H2O2 for 30 min, washed in PBS, and incubated for one h with primary antibodies against MSH2 (Dako®, FE11), MSH6 (Dako®, EP49), PMS2 (Dako®, EP51), and MLH1 (Dako®, ES05). Additionally, given that ki-67 is strongly related to the cell cycle (19) and appears to be associated with MMR proteins, [15, 22] a primary antibody for ki-67 (Dako®, MIB-1) was also used.

Samples were washed in PBS, incubated in Envision Plus HRP Anti-IgG-rabbit/mouse for 30 min (ready-to-use; monoclonal; Dako® K4065), and washed again in PBS, after which diaminobenzidine chromogen (Dako® K3469) was applied to the samples for 5 min. Harris hematoxylin was used as the counterstain (10 s), after which the specimens were dehydrated in ethanol and xylene and covered with a permanent mounting medium (Enthelam®). Colorectal carcinoma sections were used as a positive control, and the negative control and positive control were treated in parallel with an antibody diluent instead of the primary antibody.

Immunohistochemical evaluation

For evaluation of MSH2, MSH6, PMS, MLH1, and ki-67, ten fields per histological section were photographed at 400× magnification. The images were exported to ImageJ® software and counted to determine the percentage of immunostained positive cells. Brown nuclear staining of all keratinocytes cells (control group and AC group) and all cancer cells (LSCC group) was considered a positive immunoreaction. As previously

described, the imbalance of MutSα (MSH2≠MSH6) was calculated by ration between MSH2/MSH6 and imbalance of MutLα (PMS2≠MLH1) was calculated by ration between PMS2/MLH1 [15].

Statistical analysis

Data were tabulated in Microsoft Excel (Microsoft Corporation®) and exported to the Statistical Package for the Social Sciences (SPSS) software, in which the analyses were performed with a 95% confidence level.

The mean and standard deviation of the percentages of immunostaining for each of the MMR complex proteins studied, as well as the MSH2/MSH6 and PMS2/MLH1 ratios, were calculated. These data were analyzed using the Kolmogorov-Smirnov normality test and did not have a Gaussian distribution. Therefore, the comparisons were performed using Mann–Whitney and Kruskal–Wallis or Dunn tests (between-group analysis) and Wilcoxon and Spearman correlation (intragroup analysis).

Results

Sociodemographic and clinicopathological profile of removed lip lesions

Most patients were male, aged up to 65 years, living in the countryside, had no insurance, and were farmers or retired/pensioners. The lower lip was the most affected site (Table 1). Microscopically, the lip samples (n=15) showed mostly stratified squamous ortho-keratinized epithelium tissue with well-defined basal, spinous, and granular layers, occasional exocytosis, and spongiosis supported by a fibrovascular connective tissue (Figure 1).

Table 1. Sociodemographic Profile of Patients with SCC of the Lip, Actinic Cheilitis, and Lip Specimens without Microscopic Changes Evaluated Histologically for Immunohistochemical Analysis of the MMR Complex.

	Group			
	Lip	Cheilitis	LSCC	p-Value
Total	15 (100.0%)	30 (100.0%)	45 (100.0%)	-
Sex				
Female	5 (33.3%)	15 (50.0%)	11 (24.4%)	0.074
Male	10 (66.7%)	15 (50.0%)	34 (75.6%)	
Age				
Up to 65	7 (46.7%)	14 (46.7%)	24 (53.3%)	0.819
>65	8 (53.3%)	16 (53.3%)	21 (46.7%)	
Origin				
Countryside	11 (73.3%)	16 (53.3%)	27 (60.0%)	0.564
Metropolitan area	1 (6.7%)	7 (23.3%)	6 (13.3%)	
Capital	3 (20.0%)	7 (23.3%)	12 (26.7%)	
Living in				
Countryside	11 (73.3%)	16 (53.3%)	27 (60.0%)	0.564
Metropolitan area	1 (6.7%)	7 (23.3%)	6 (13.3%)	
Capital	3 (20.0%)	7 (23.3%)	12 (26.7%)	
Service entrance				
Public Health System	15 (100.0%)	27 (93.1%)	37 (92.5%)	0.558
Health Insurance/Private	0 (0.0%)	2 (6.9%)	3 (7.5%)	
Profession				
Farmer	3 (37.5%)	7 (38.9%)	12 (46.2%)	0.548
Retired/Pensioner	4 (50.0%)	11 (61.1%)	11 (42.3%)	
Other professions	1 (12.5%)	0 (0.0%)	3 (11.5%)	
Topography				
Lower lip	3 (20.0%)	4 (13.3%)	7 (15.5%)	0.844
Upper lip	12 (80.0%)	26 (86.7%)	38 (84.5%)	
Tumor thickness				
=<2 mm	-	-	30 (66.7%)	-
>2 mm	-	-	15 (33.3%)	
Depth of invasion				
=<5 cm	-	-	12 (26.7%)	-
5-10 cm	-	-	18 (40.0%)	
>10 cm	-	-	15 (33.3%)	

*p<0.05, Fisher's exact test or Pearson's chi-square test (n, %); LSCC, squamous cell carcinoma.

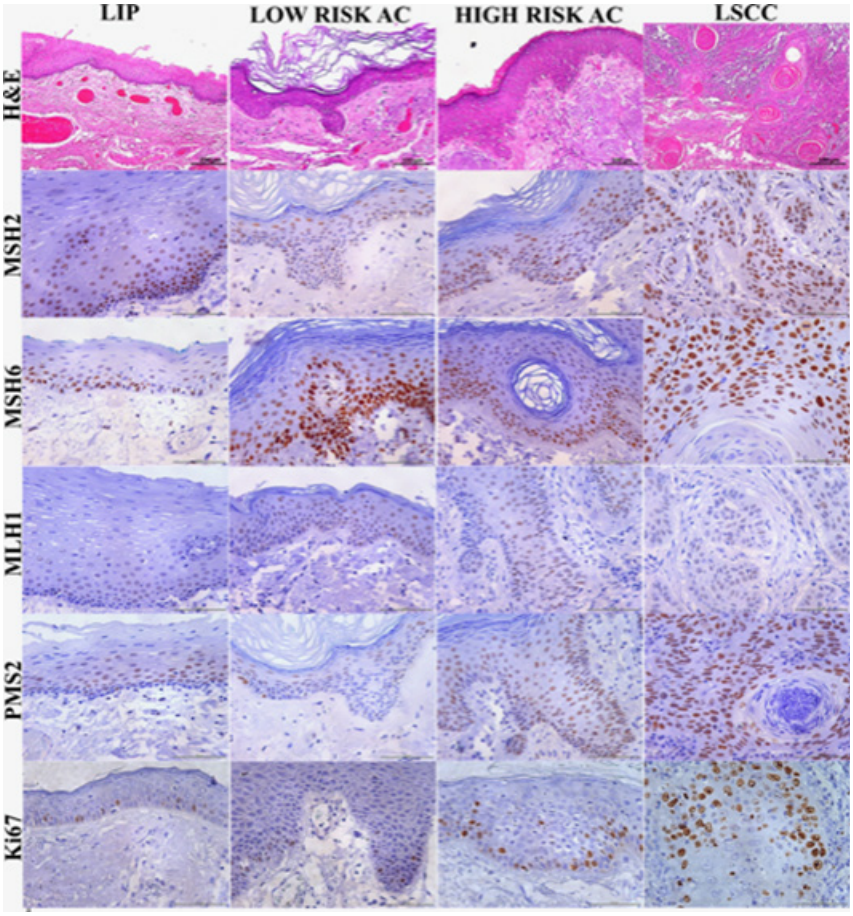

Figure 1. Representative Images from H&E and Immunostaining for MSH2, MSH6, PM2, MLH1 and ki6-7 photomicrographs of NLE, AC with low dysplasia, AC with high dysplasia and LSCC. Magnification of H&E = 200×; Magnification of IHC = 400×; The percentage of MSH2 positive cells was reduced in the AC and increased in the LSCC. The MSH6, PMS2, and ki-67 immunostained cells increased in AC and LSCC, and MLH1 immunostaining did not differ between groups.

AC samples (n=30) exhibited atrophic stratified squamous keratinized epithelium tissue with four to six layers of squamous cells, disorganization of the basal and spinous layers, significant nuclear hyperchromatism cellular pleomorphism, and were parakeratinized. The supporting connective tissue showed solar elastosis throughout the lesions and occasional mononuclear inflammatory cells (Figure 1). Most AC patients had low-risk dysplasia (n=19).

The LSCC samples (n=45) showed proliferation of islands and strands of cells of epidermoid origin, organized, sometimes in nests, exhibiting intense cellular and nuclear pleomorphism, keratin pearls, typical and atypical mitotic figures, and intense mixed inflammatory infiltrate (Figure 1). Of the LSCCs evaluated, 29 (64.4%) were low-grade,5 (11.1%) showed vascular invasion, and 12 (26.7%) showed perineural invasion (Table 2). Most patients were men (n=34, 75.5%), aged <65 years (n=24, 53%), and the average age of 64.7±12.2 years. The most prevalent tumor TNM stage was T1/2 (n=30, 66.7%), N0 (n=29, 64.4%), and M0 (n=42, 93.3%), and recurrence was observed in only three (6.7%) of the patients (Table 3). The median of follow up time was 13 months (range = 3-96 months).

Immunostaining profile for MutSα and MutLα in lip lesions

All tissues showed positive immunostaining for MMR proteins. Immunostaining was concentrated in the basal and parabasal layers in the lip, in the basal, paranasal, and spinous layers in the AC, and in the tumor islands and nests in LSCC (Figure 1).

Immunostaining for MSH2, MSH6, MLH1, and ki-67 was significantly higher in AC and LSCC tissues ($p<0.001$, $p<0.001$, $p=0.040$, and $p=0.004$, respectively). MSH2 immunostaining was significantly higher in LSCC ($p<0.001$). An imbalance was observed in MutSα, with higher expression of MSH2 in the lip ($p=0.043$), and the opposite relationship in AC ($p=0.005$), which was also noted in the MSH2/MSH6 ratio ($p<0.001$). The mean MSH2/MSH6 ratio was lower in the AC group ($p=0.028$) (Figure 1; Table 2).

Influence of clinicopathological characteristics of SCC of the lip on the immunostaining of MMR complex proteins

Lower expression of MSH2 compared to MSH6 was observed in AC with low-risk dysplasia ($p=0.018$). Immunostaining for MSH6 was significantly lower in patients with AC with high-risk dysplasia ($p=0.024$). The presence of vascular invasion in LSCC was associated with a lower mean MSH6 ($p=0.035$). In LSCC depth of

Table 2. Immunostaining Profile for MutSα and MutLα Heterodimers in the Removed Lip Lesions and Influence of Microscopic Findings

	MutSα			MutLα			Ratio			
	MSH2 (%)	MSH6 (%)	p-Value[a]	PMS2 (%)	MLH1 (%)	p-Value[a]	MSH2/MSH6	PMS2/MLH1	p-Value[a]	Ki-67 (%)
Lip Lesions										
NLE (n=15)	63.23±13.79	51.69±13.87	0.043	47.18±12.12	44.67±9.34	0.5	1.26±0.32	1.08±0.32	0.593	1.20±0.86
Cheilitis (n=30)	49.45±19.41*	77.04±8.85*	0.005	57.68±12.25	60.69±13.14*	0.424	0.67±0.24*	0.98±0.24	0.028	9.82±4.65*
LSCC (n=45)	75.82±10.84*†	74.27±11.28*	0.296	60.41±20.46	60.35±12.12*	0.157	1.02±0.13*†	1.05±0.18	0.387	9.10±9.59*
p-Value[b]	<0.001	<0.001		0.106	0.04		<0.001	0.461		0.004
Histological grading of dysplasias										
Low risk (n=19)	53.75±14.83	80.46±7.74	0.018	55.86±12.69	61.46±13.50	0.241	0.72±0.09	0.95±0.24	0.046	8.82±4.37
High risk (n=11)	36.53±29.30	68.51±4.89*	0.109	64.95±8.13	52.24±0.00	1	0.53±0.43	1.26±0.10	0.025	11.80±4.95
p-Value[b]	0.309	0.024		0.194	0.469		0.732	0.206		0.21
Histological grading of LSCC										
Low (n=29)	74.42±9.99	74.66±12.39	0.741	58.83±19.72	55.45±15.87	0.094	1.00±0.11	1.09±0.22	0.177	6.54±12.50
High (n=17)	78.12±12.13	73.69±9.79	0.182	63.18±22.32	63.41±21.97	0.722	1.04±0.15	1.00±0.08	0.646	6.75±8.47
p-Value[b]	0.247	0.973		0.432	0.181		0.278	0.208		0.964
Lymphovascular invasion (LSCC)										
No (n=40)	76.62±10.18	75.81±11.22	0.6	62.96±19.24	61.00±17.16	0.13	1.00±0.10	1.06±0.19	0.131	6.64±11.68
Yes (n=5)	70.70±14.69	65.00±6.57*	0.498	46.13±23.45	45.66±22.15	0.684	1.09±0.23	1.01±0.11	0.498	6.26±8.65
p-Value[b]	0.45	0.035		0.16	0.096		0.071	0.603		0.957
Perineural invasion (LSCC)										
No (n=32)	75.93±10.48	75.69±12.03	0.543	62.69±20.38	60.47±16.27	0.212	1.00±0.11	1.04±0.18	0.349	7.27±12.62
Yes (n=13)	75.59±12.03	70.72±8.63	0.386	55.19±20.75	55.00±22.51	0.575	1.05±0.16	1.07±0.20	0.767	4.79±6.74
p-Value[b]	0.922	0.29		0.347	0.509		0.481	0.963		0.604
Tumor thickness										
=<2 mm (n=30)	74.23±12.89	81.09±12.15	0.345	52.59±24.15	47.00±22.29	0.686	0.91±0.16	1.02±0.13	0.144	8.19±10.77
>2 mm (n=15)	75.10±10.41	70.87±9.05	0.043	55.97±22.02	49.30±23.14	0.068	1.06±0.04	1.36±0.18	0.068	20.57±21.93
p-Value[b]	0.808	0.167		0.85	0.602		0.028	0.014		0.3
Depth of invasion										
=<5 cm (n=12)	76.33±1.56	88.28±22.15	0.606	62.32±14.43	48.15±21.45	1	0.89±0.24	1.17±0.23	1	6.44±1.48
5-10 cm (n=18)	73.40±15.67	78.22±7.93	0.316	48.70±27.51	47.00±22.29	0.651	0.92±0.14	1.02±0.13	0.137	1.83±0.35
>10 cm (n=15)	75.10±10.41	70.87±9.05	0.027	55.97±22.02	49.30±23.14	0.007	1.06±0.04	1.36±0.18*	0.07	7.31±7.46
p-Value[b]	0.955	0.206		0.784	0.877		0.203	0.013		0.539

[a], Test Wilcoxon; [b], Test Kruskal-Wallis/Dunn, *p<0.05 versus lip, †p<0.05 versus cheilitis (media ±SD). Tumor thickness >2cm showed a less im

Table 3. Influence of Clinical Features on the Immunostaining Profile for MutSα and MutLα Heterodimers in LSCC

	MutSα			MutLα			Ratio			
	MSH2 (%)	MSH6(%)	p-Value[a]	PMS2 (%)	MLH1 (%)	p-Value[a]	MSH2/MSH6	PMS2/MLH1	p-Value[a]	Ki-67 (%)
Sex										
Female (n=11)	75.45±8.97	70.75±8.46	0.249	59.36±20.45	55.00±18.92	0.063	1.07±0.11	1.11±0.22	0.5	11.61±22.42
Male (n=34)	75.92±11.44	75.15±11.84	0.346	60.70±20.86	59.56±18.71	0.79	1.00±0.13	1.04±0.17	0.615	6.37±8.76
	0.74	0.343		0.792	0.637		0.202	0.554		0.364
Age										
Up to 65 (n=24)	51.08±8.15	72.07±8.87	0.394	57.55±9.10	64.46±19.92	0.649	0.70±0.10	1.00±0.25	0.722	5.88±7.46
>65 (n=21)	48.90±22.36	80.78±7.22	0.557	57.72±13.61	59.44±11.45	0.215	0.65±0.28	0.97±0.26	0.535	8.83±15.41
p-Valueb	0.447	0.597		0.943	0.937		0.8	0.792		0.491
T										
T1/2 (n=30)	74.23±12.89	81.09±12.15	0.345	52.59±24.15	47.00±22.29	0.686	0.91±0.16	1.02±0.13	0.144	8.19±10.77
T3/4 (n=15)	75.10±10.41	70.87±9.05	0.043	55.97±22.02	49.30±23.14	0.068	1.06±0.04	1.36±0.18	0.068	20.57±21.93
p-Value[b]	0.808	0.167		0.85	0.602		0.028	0.014		0.3
N										
N0 (n=29)	78.66±4.27	82.26±11.36	0.917	60.46±15.18	60.63±12.63	0.465	0.97±0.14	1.03±0.18	0.109	6.82±10.20
N+ (n=16)	70.86±15.24	72.52±10.71	0.463	49.95±27.40	41.43±24.26	0.075	0.97±0.14	1.24±0.23	0.046	27.43±20.95
p-Value[b]	0.606	0.153		0.423	0.201		1	0.286		0.039
M										
M0 (n=42)	74.07±8.04	77.09±14.50	0.753	49.04±15.84	44.71±21.50	0.273	0.98±0.13	1.22±0.35	0.109	6.76±5.80
M1 (n=3)	67.64±20.63	72.28±13.81	1	54.57±37.63	45.61±30.87	0.109	0.93±0.20	1.16±0.11	0.109	7.89±8.91
p-Value[b]	0.732	0.732		0.606	0.881		0.796	0.724		0.885
Recurrence										
No (n=47)	76.16±9.89	73.87±11.42	0.171	61.45±18.84	59.91±17.05	0.2	1.03±0.12	1.05±0.19	0.648	9.09±9.58
Yes (n=3)	71.96±21.85	78.48±10.58	0.593	50.08±37.10	45.63±30.88	0.593	0.90±0.17	1.07±0.10	0.285	8.74±7.70
p-Value[b]	0.956	0.409		0.661	0.385		0.188	0.519		0.77

[a],Wilcoxon head; [b], Man-Whitney head, *p<0.05 (mean ±SD). The three recurrences were local recurrences. No distant metastasis were showed in this sample.

Table 4. Correlation between the MMR Complex Proteins in the Removed Lesions of the Lip

	MSH2	MSH6	PMS2	MLH1	Ki-67
Lip					
MSH2	-	p=0.432 (r=0.357)	p=0.285 (r=0.600)	p=0.800 (r=-0.200)	p=0.397 (r=0.429)
MSH6	-	-	p=0.432 (r=0.357)	p=0.208 (r=0.600)	p=0.876 (r=-0.071)
PMS2	-	-	-	p=0.285 (r=0.600)	p=0.188 (r=-0.700)
MLH1	-	-	-	-	p=0.544 (r=0.314)
Ki-67	-	-	-	-	-
Cheilitis					
MSH2	-	p=0.038 (r=0.661)	p=0.803 (r=0.091)	p=0.265 (r=0.417)	p=0.460 (r=0.283)
MSH6	-	-	p=0.790 (r=0.091)	p=0.865 (r=0.067)	p=0.574 (r=-0.191)
PMS2	-	-	-	p=0.298 (r=0.345)	p=0.593 (r=0.182)
MLH1	-	-	-	-	p=0.637 (r=0.183)
Ki-67	-	-	-	-	-
LSCC					
MSH2	-	p<0.001 (r=0.753)	p<0.001 (r=0.691)	p<0.001 (r=0.810)	p=0.544 (r=0.133)
MSH6	-	-	p=0.002 (r=0.526)	p<0.001 (r=0.689)	p=0.697 (p=0.086)
PMS2	-	-	-	p<0.001 (r=0.872)	p=0.384 (r=0.206)
MLH1	-	-	-	-	p=0.352 (r=0.220)
Ki-67	-	-	-	-	-

a, Spearman correlation.

invasion >10cm showed less immunostained cells for MSH6 than MSH2 (p=0.027) and MLH1 than PMS2 (p=0.007). A significant imbalance in PMS2/MLH1 ratio was showed in LSCC with a depth of invasion >10cm than <10cm depth invasion (p=0.013) (Table 2).

T3/T4 patients showed higher mean values of MSH2/MHS6 and PMS2/MLH1 ratio compared to T1/T2 (p=0.028, p=0.014), whereas N + patients showed higher mean values of PMS2/MLH1 than MSH2/MHS6 (p=0.046). N+ patients showed higher immunostaining for ki-67 than did N0 patients (p=0.039) (Table 3).

MMR complex proteins correlate in SCC of the lip

LSCC exhibited a greater number of positive correlations, with all MMR

proteins correlated with each other. AC presented less statistically significant findings, with positive correlations only between MSH2 and MSH6 (p=0.038). No correlations were found in lip samples (Table 4).

Discussion

Recent studies have shown that low expression of MMR components has been linked to several types of cancers [3, 10, 15, 16, 23-25]. The MutSα complex, particularly hMSH2, is crucial for post-replication DNA correction [26]. In oral epithelium, hMSH2's role in mismatch identification is vital, and its inefficiency contributes to potentially malignant lesions [13]. Immunohistochemical studies show lower hMLH1 and hMSH2 expression in more dysplastic lesions of lower parturition squamous cell carcinoma [12]. Variable protein expression in different dysplasia degrees adds complexity to genetic regulation [27]. Reduced hMSH2 expression is associated with factors like smoking, suggesting its involvement in oral carcinogenesis [28]. Animal model experiments reveal that MSH2 overexpression correlates with genetic instability, promoting UVB radiation-induced carcinogenesis in the skin [29].

Alterations in MSH6 within the MutSα complex are crucial in carcinogenesis [11, 17, 24]. While previous studies noted a loss of hMSH6 expression in oral dysplasia and SCC [25], the present study on lip lesions found increased MSH6 expression due to repair attempts. Overexpression of MSH6 disrupts mechanisms preventing recombination between DNA sequences, interacting with DNA [30]. Co-overexpression of MSH2 and MSH6 leads to genome instability [10], potentially promoting cancer progression [31].

The expression of MSH6 was remarkably similar to that of MLH1. There was an increased expression in AC and lip SCC, with no modification in the expression of MSH2 [24]. A low expression of MMR proteins, especially MLH1 and MSH2, is associated with oral carcinogenesis [10]. Although we have observed that cheilitis with high-grade dysplasia and LSCCs with lymphovascular invasion have a loss of MSH6 expression, the process differs in lip carcinogenesis. Apparently, an imbalance of MutLα proteins could be due to a compensatory mechanism for poor functional performance in the repair attempt [24, 25].

Because malignant cells remain in a continuous division, they are permanently challenged with active DNA repair. As a result, the performance of the MMR complex proves to be limited once these corrections can keep up with the rate of bipartition errors or the corrections are deficient, resulting in microsatellite instability due to failure to repair incorrect insertion/deletion pairs [25, 31].

With the results of the present study we have also

shown that in LSCC, vascular invasion is associated with a reduction in the number of MSH6-immunopositive cells and staging with an imbalance in both MutSα and MutLα complexes. A previous study evaluating microsatellite instability in gastric cancer tumors with nodal metastasis also showed loss of MMR protein expression, [15] suggesting that MMR deficiency is also associated with oral carcinogenesis progression and greater aggressiveness in tumors [11]. These findings seem to be independent of sex and age [11].

In previous studies using oral and oropharyngeal SCC samples, the MMR complex directly influenced the prognosis in specific subpopulations [15, 16]. We can observe that patients who survived had a lower mean immunostaining. Nevertheless, because lip tumors have a good prognosis [31, 32], a small sample of recurrent tumors makes more solid conclusions impossible. Correlations between MMR proteins followed tumor progression, probably due to DNA mismatches that trigger an MMR system response during malignant transformation [25, 31]. However, these proteins did not follow ki-67 expression, similar to what was observed in oropharyngeal tumors [15].

Additionally, the analysis method of counting immunostaining cells is still subjective and time-consuming, which makes it unfeasible to carry out large volumes of analyzes. However, despite computer-assisted tumor grading of quantification of immunostaining cell density is not yet robust enough for all markers [33]. These methods have evolved over the last 20 years, but pathologist-machine agreement values are still limited [34, 35]. and still need validation by pathologists [36].

In colorectal cancer MMR alterations is directly associated with prognosis and indication to chemotherapy and the standard treatment of these tumors is commonly associated with platinum-based chemotherapy [37] similar to head and neck cancer [38]. So, in altered LSCC related to MMR the addition of chemotherapy can improve the treatment and reduce risk of treatment. However, the major disvantage is the increase in cousts of exams due addition of MSI tests for LSCC [39].

The retrospective nature of this study may limit its level of clinical evidence and there were two main limitations: the lack of MSI analysis (difficult to perform in paraffin blocks) and the sample size, which was limited due to the generally good prognosis of LSCC; a much larger sample would be necessary to obtain an association between immunostaining and recurrence rates. Additionally, TMA use is a significant limitation due possible differences between cores and complete sample, despite previous studies showed that this method be effective in immunohistochemistry assays [40]. In oral squamous cell carcinoma, when TMA is used, a 1 core underestimates immunostaining, but 2 core samples have the same sensitivity than whole sections [41]. So, we used 2 core samples to minimize underestimation of immunohistochemistry analysis.

This is the first study to address the immunostaining for all MMR complex proteins in the three stages of LSCC progression and clinical-pathological features.

In conclusion, there is increased activity and inter-association between these proteins in tumors, but their loss of expression significantly worsens the prognosis.

Author Contribution Statement

All authors contributed equally in this study.

Acknowledgements

We would like to thank the Hospital Haroldo Juaçaba (HHJ) – Cancer Institute of Ceará for the contributions to this research, for providing the infrastructure for the execution of this study.

Ethic Approval

This research was submitted to the Research Ethics Committee of the HHJ/ICC, observing the norms that regulate research with human beings, of the Brazilian National Health Council under resolution 466/12. The opinion number was 2.191.839

Conflict of interest

None to declarate.

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