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Overexpression of MutSα Complex Proteins Predicts Poor Prognosis in Oral Squamous Cell Carcinoma

Vivian Petersen Wagner, DDS, Liana Preto Webber, DDS, MS, Gabriela Salvadori, DDS, Luise Meurer, MD, PhD, Felipe Paiva Fonseca, DDS, PhD, Rogério Moraes Castilho, DDS, PhD, Cristiane Helena Squarize, DDS, PhD, Pablo Agustin Vargas, DDS, PhD, and Manoela Domingues Martins, DDS, PhD

Abstract: The DNA mismatch repair (MMR) system is responsible for the detection and correction of errors created during DNA replication, thereby avoiding the incorporation of mutations in dividing cells. The prognostic value of alterations in MMR system has not previously been analyzed in oral squamous cell carcinoma (OSCC).

The study comprised 115 cases of OSCC diagnosed between 1996 and 2010. The specimens collected were constructed into tissue microarray blocks. Immunohistochemical staining for MutSα complex proteins hMSH2 and hMSH6 was performed. The slides were subsequently scanned into high-resolution images, and nuclear staining of hMSH2 and hMSH6 was analyzed using the Nuclear V9 algorithm. Univariable and multivariable Cox proportional hazard regression models were performed to evaluate the prognostic value of hMSH2 and hMSH6 in OSCC.

All cases in the present cohort were positive for hMSH2 and hMSH6 and a direct correlation was found between the expression of the proteins ($P < 0.05$). The mean number of positive cells for hMSH2 and hMSH6 was 64.44 ± 15.21 and 31.46 ± 22.38, respectively. These values were used as cutoff points to determine high protein expression. Cases with high expression of both proteins simultaneously were classified as having high MutSα complex expression. In the multivariable analysis, high expression of the MutSα complex was an independent prognostic factor for poor overall survival (hazard ratio: 2.75, $P = 0.02$).

This study provides a first insight of the prognostic value of alterations in MMR system in OSCC. We found that MutSα complex may constitute a molecular marker for the poor prognosis of OSCC.

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Abbreviations: HNPCC = hereditary nonpolyposis colon cancer, MMR = DNA mismatch repair, OSCC = oral squamous cell carcinoma, SD = standard deviation, TMA = tissue microarray.

INTRODUCTION

The multistep process of oral carcinogenesis depends on the accumulation of genetic and epigenetic alterations, leading to genomic instability, cellular transformation, and tumor progression.[1] The DNA damage repair mechanisms are responsible for the maintenance of genome integrity.[2] Germline mutations of DNA damage repair genes as well as the epigenetic-driven loss of function result in the enhanced incorporation of mutations and, consequently, genomic instability. The DNA mismatch repair (MMR) system is responsible for the detection and correction of errors created during DNA replication, thereby avoiding the incorporation of mutations in dividing cells. This pathway eliminates severely damaged and genomically instable cells and thereby plays as important role in the prevention of short-term mutagenesis and long-term tumorigenesis.[2,3]

The DNA MMR system is composed of 3 related, yet distinct, proteins subunits: MutSα (hMSH2+hMSH6), MutSβ (hMSH2+hMSH3), and MutLα (hMLH1+hPMS2). The MutSα complex is continuously expressed during all phases of the cell cycle and is capable of movement along the contours of homoduplex DNA. hMSH2 and hMSH6 are required in the initiation of the MMR pathway. Moreover, the MutSα complex can interact with many downstream proteins involved in induced cell cycle arrest, apoptosis, chromatin remodeling, and other DNA repair pathways.[4] The MMR system has been widely studied in the past decade due to its association with several diseases and conditions, including hereditary nonpolyposis colon cancer (HNPCC).[5] More recently, an increased number of cancers have been associated with defects in the MMR mechanism, including colon, endometrium, and prostate cancer.[6,7]

Previous studies have suggested the loss of expression of MMR-related proteins, especially hMLH1, during the process of oral carcinogenesis.[8–15] However, most of these studies focused on potentially malignant oral disorders. Moreover, the prognostic value of MMR proteins in OSCC remains unknown. In esophageal squamous cell carcinoma, Uehara et al[7] found that the absence of the immunohistochemical expression of hMLH1 and hMSH2 was associated with poor

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From the Department of Oral Pathology, School of Dentistry, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil (VPW, LPW, MDM); Experimental Pathology Unit, Hospital de Clínicas de Porto Alegre, Porto Alegre, Rio Grande do Sul, Brazil (VPW, LPW, LM, MDM); Institute of Oral Biology, Faculty of Dentistry, University of Oslo, Norway (GS); Department of Oral Diagnosis, Piracicaba Dental School, University of Campinas, Piracicaba, São Paulo, Brazil (FPF, PAV); and Laboratory of Epithelial Biology, Department of Periodontics and Oral Medicine, University of Michigan, School of Dentistry, Ann Arbor, MI (RMC, CHS).
Correspondence: Manoela Domingues Martins, Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul Rua Ramiro Barcelos, 2492, sala 503, CEP 90035–003, Santana, Porto Alegre RS, Brazil (e-mail: manomartins@gmail.com).
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survival rates. Recently, hMSH6 overexpression has been shown to be associated with a poor prognosis in cases of melanoma[16] and osteosarcoma[17] and correlated to tumor progression during chemotherapy in malignant pleural mesothelioma.[18]

Current literature provides some insights regarding the role of the MMR system in oral carcinogenesis. However the clinical significance of these phenomena remains unclear. To the best of our knowledge, there are no data regarding the possible prognostic value of MMR proteins for cases of OSCC. Moreover, hMSH6 expression has never been evaluated in OSCC, yet both hMSH2 and hMSH6 are required for the initiation of the MMR pathway. Thus, the aim of the present study was to access the value of MutSα complex proteins hMSH2 and hMSH6 in OSCC and correlate the findings with clinical-pathological aspects and overall survival rates.

METHODS

Study Population

One hundred fifteen stored tissue blocks of OSCC diagnosed between 1996 and 2010 by the Pathology Laboratory of the Porto Alegre University Hospital in Brazil were included in this retrospective study (Human Research Ethics Committee approval: 49942215.2.0000.5327). Cases with a histopathological diagnosis of OSSC on the tongue, floor of the mouth, lip, buccal mucosa, and palate were randomly selected. The medical records were manually evaluated and data were collected on demographic characteristics, risk factors, clinical presentation, treatment, and outcomes. Pack-years of smoking were calculated using the following formula: ([mean number of cigarettes smoked per day/20] × number of years smoked). The follow-up period was defined as the date of diagnosis until the last visit to the hospital or date of death. Only cases with at least 70% of complete information in the medical records and an adequate amount of material for the analysis of specimens were included. Histological sections were graded based on the criteria described by Bryne et al.[19]

Tissue Microarray Construction

The specimens retrospectively collected were constructed into tissue microarray (TMA) blocks for immunohistochemical analysis. TMA construction was performed as described elsewhere.[20] Briefly, tumor areas of the invasive front of the tumor were selected and marked on hematoxylin-eosin-stained sections using an objective marker (Nikon Corp, Tokyo, Japan). The slide was then overlaid on the original paraffin block to determine the corresponding area to be used. A manual tissue arrayer (Sakura Co, Japan) was used and 3 representative cylindrical cores from the invasive front measuring 2.0 mm in diameter were taken from each tissue block of OSCC and arranged sequentially in a ready-to-use recipient paraffin block (Sakura Co, Japan). Three cores of normal mucosa were inserted in the left upper corner of each recipient block for orientation. A map specifying the exact position of each case was prepared to facilitate the interpretation of the immunohistochemical results.

Immunohistochemistry

For immunohistochemical staining, the samples were sectioned into 3-μm sections and placed on silanized slides. The slides were de-paraffinized in xylene and hydrated in descending grades of ethanol. Antigen retrieval was performed prior to incubation of the primary antibodies. The primary antibodies, sources, clone, antigen retrieval, dilutions, and incubation times were as follows: hMSH2 (Santa Cruz, polyclonal, low pH solution in a water bath at 90°C for 18 h, 1:100, 18 h) and hMSH6 (Cell Marque, 44, low pH solution in a water bath at 90°C for 18 h, 1:50, 18 h). The sections were then incubated with diaminobenzidine tetrahydrochloride (DAB, Novocastra, Newcastle, UK) and counterstained with Mayer's hematoxylin. Negative controls were obtained by replacing the primary antibodies with nonimmune serum. The positive control used for both proteins was benign colonic tissue.

Digital Analysis

The immunohistochemical slides were subsequently scanned into high-resolution images using the Aperio Scanscope CS Slide Scanner (Aperio Technologies Inc, Vista, CA). All digital images obtained in .svs format were visualized using the ImageScope software (Aperio Technologies Inc., Vista, CA). MSH2 and MSH6 nuclear staining was analyzed using the Nuclear V9 algorithm (Aperio Technologies Inc, Vista, CA) with the following input parameters: averaging radius: 0.9; curvature threshold: 2.5; lower threshold: 0; upper threshold: 230; minimum nuclear size: 22; maximum nuclear size: 165; minimum roundness: 0.3; minimum compactness: 0.1; minimum elongation: 0.2; clear area objective: 240; and an intensity threshold ranging from 0 to 230, in which strong staining was considered from 0 to 185 and weak staining was from 185 to 230. At least 1000 cells were quantified in 10 hotspot areas of each case and the percentage of positive cells was determined.

Statistical Analysis

All clinical and immunohistochemical data were analyzed with the aid of the SPSS (IBM Corporation, Armonk, NY), version 18.0. Differences between groups were evaluated using the Mann-Whitney test. Spearman's correlation coefficients were calculated to determine the correlation of hMSH2 and hMSH6 expression. Univariable and multivariable Cox proportional hazard regression models were performed to evaluate poor prognosis predictors in OSCC. The assumption of proportional hazards was verified for all variables entering the model. Independent variables with $P < 0.25$ in univariable models were incorporated into the multivariable model. The stepwise backward method was used to achieve a final model and maintenance of variables was determined by $P < 0.10$. Kaplan-Meier cumulative survival curves were constructed. For all tests, a $P < 0.05$ was considered indicative of statistical significance.

RESULTS

Cohort of OSCC Patients Used in Study

A cohort of 115 patients derived from the Porto Alegre University Hospital in Brazil was used in this study (Table 1). The majority of the patients were Caucasian (92.1%) compared to black and non-otherwise specified patients. The proportion of males was higher (87%) in comparison to females. The majority of the patients had history of smoking, with over 60% of the population current smokers, while 13.9% were former smokers. Nonsmokers represented a smaller proportion (11.3%). The majority of patients were diagnosed in an advanced clinical stage (65.2%). The tumors were graded in histopathological grades II and III in over 70% of patients. The follow-up period

TABLE 1. Clinical-Pathological Aspects of Patients With OSCC

Demographic/Clinical Characteristics	OSCC (n = 115)
Age	60.89 (±10.80)
Gender	
Male	100 (87%)
Female	15 (13%)
Ethnicity	
Caucasian	106 (92.1%)
Black	8 (7%)
Missing	1 (0.9%)
Residence	
Urban	96 (83.5%)
Rural	17 (14.8%)
Missing	2 (1.7%)
Tobacco habits	
User	70 (60.9%)
Former user	16 (13.9%)
Non-user	13 (11.3%)
Missing	16 (13.9%)
Pack-years	39.19 (23.90)
Clinical aspects	
Ulcer	93 (79.9%)
Spot/plaque/nodule	10 (9.7%)
Missing	12 (10.4%)
Pain	
Yes	70 (60.9%)
No	26 (22.6%)
Missing	19 (16.5)
Size	
T1/T2	40 (34.8%)
T3/T4	67 (58.3%)
Missing	8 (7%)
Nodal metastasis	
Yes	56 (48.7%)
No	53 (46.1%)
Missing	6 (5.2%)
TNM	
I/II	34 (29.6%)
III/IV	75 (65.2%)
Missing	6 (5.2%)
Recurrence	
Yes	38 (33%)
No	73 (63.5%)
Missing	4 (3.5%)
Histopathological grade*	
Grade I	32 (27.8%)
Grade II/III	83 (72.2%)
Evolution	
Living	79 (68.7%)
Deceased	33 (28.7%)
Missing	3 (2.6%)

OSCC = oral squamous cell carcinoma.
*According to Bryne et al.[19]

ranged from 1 to 140 months (mean ± standard deviation (SD): 43.21 ± 35.85). During follow-up, 33% of patients presented recurrence, with a mean ± SD time to recurrence of 19.44 ± 14.40 months and 29.5% of patients (n = 33) deceased because of the tumor or associated causes.

Active MutSα Complex in OSCC

All cases included in the analysis presented positive cells for both hMSH2 and hMSH6 (Figure 1A). The mean number of positive cells for hMSH2 and hMSH6 was 64.44 ± 15.21 and 31.46 ± 22.38, respectively. The means were used as the cutoff points to determine low and high expression of hMSH2 and hMSH6 (Figure 1B and C). High hMSH2 and hMSH6 expression was found in 47 (59.1%) and 61 (46.5%) cases, respectively. Thirty-two cases (27.8%) exhibited high hMSH2 and hMSH6 expression simultaneously. These cases were classified as exhibiting high MutSα expression. Figure 2 and Table 2 displays the expression of hMSH2 and hMSH6 according to the main clinical-pathological aspects. Both proteins demonstrated homogenous distribution regarding tobacco habits, presence of nodal metastasis, clinical stage, recurrence, and histopathological grade. An association was found between hMSH2 expression and tumor size, in which tumors with a higher T status (T3/T4) had lower hMHS2 expression ($P = 0.03$, Mann-Whitney test).

Direct Correlation Between hMSH2 and hMSH6 Expression

Once we found that all tumors from the 115 patients were positive for hMSH2 and hMSH6, we decided to determine whether the both markers were correlated. Indeed, we found that hMSH2 expression levels in OSCC were significantly correlated with the levels of hMSH6, as demonstrated by the Spearman correlation coefficient of 0.38 ($P < 0.05$) (Figure 3).

Higher Expression of the MutSα Complex As a Predictive Factor of Poor Prognosis in OSCC

The univariable analysis revealed that the presence of pain at diagnosis, a higher T status (T3/T4), advanced clinical stage (TNM III/IV), recurrence, higher degrees of malignancy (Bryne's grade II/III), hMSH6 and hMutSα overexpression were associated with poor survival rates (Table 3). Recurrence, clinical stage, and MutSα overexpression remained significantly associated with poor survival rates in the multivariable analysis. The Kaplan-Meier overall survival curve demonstrated that the first 25 months after diagnosis were associated with the most pronounced decline in cumulative survival (Figure 4A). Patients with MutSα overexpression had poor survival rates during the follow-up period in comparison to patients with lower MutSα expression (Figure 4B).

DISCUSSION

In recent decades, no absolute gain has been achieved in OSCC survival rates,[21] and this tumor remains responsible for more than 120,000 deaths per year worldwide.[22] Alterations in the MMR system have previously been described in potentially malignant oral disorders and OSCC.[8–15] However, the prognostic value of these alterations has not previously been analyzed. In the present study, immunohistochemical analysis of MutSα complex proteins hMSH2 and hMSH6 was performed for 115 cases of OSCC. Our main finding revealed that MutSα complex overexpression was associated with poor overall survival in patients with OSCC.

The DNA MMR system corrects base mismatches that occur during replication.[2] The MutSα complex is composed of 1 molecule of hMSH2 and 1 molecule of hMSH6 and is capable of recognizing base/base mismatches and short insertion/deletion loops.[4] In the early 1990s, defects in the MMR system were

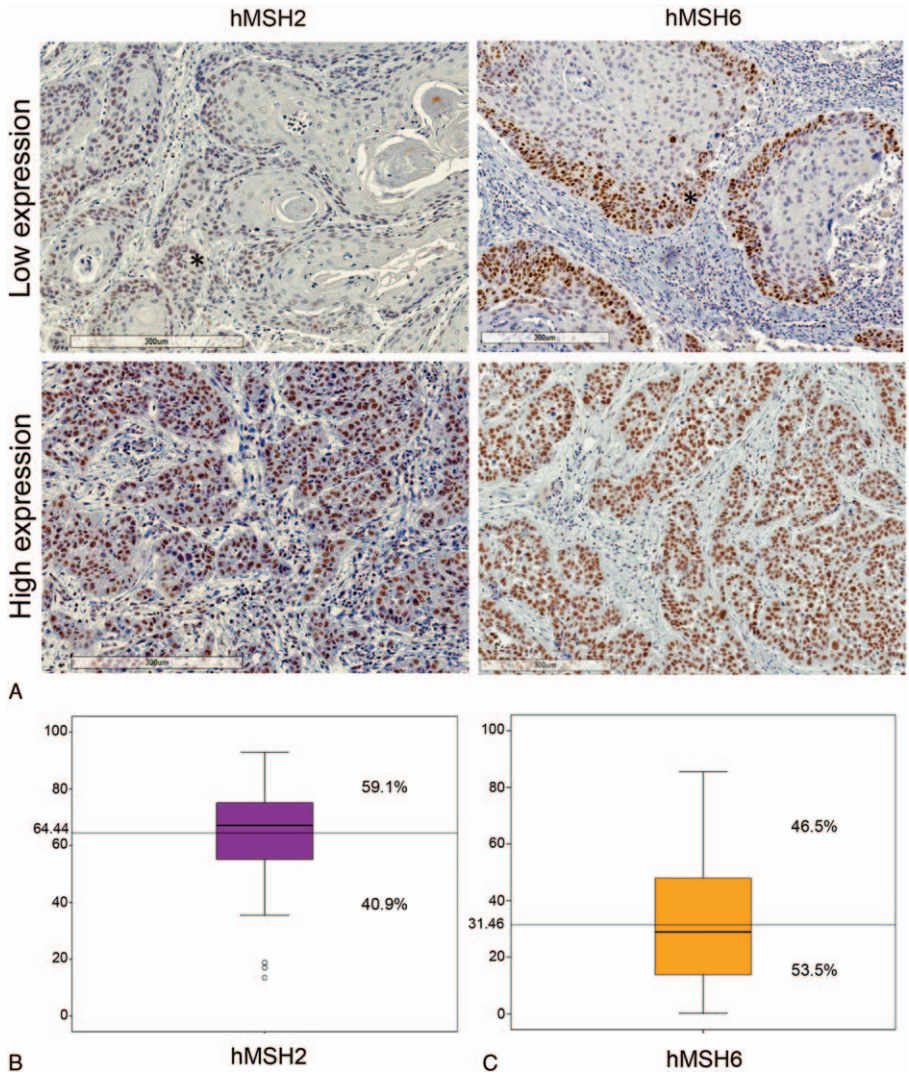

FIGURE 1. MutSα complex expression in OSCC. A, Representative examples of hMSH2 and hMSH6 expression in OSCC. hMSH2 and hMSH6 presented nuclear staining in tumor cells. Note that in low expression cases, staining was restricted in the periphery of tumor islands (*), whereas in high expression cases, staining was homogenously distributed throughout the tumor. Box plots of (B) hMSH2 and (C) hMSH6 demonstrating the distributing of proteins expression in the present OSCC cohort. For each protein, the main value (horizontal line) was used as a cutoff point to determine cases of low and high expression. OSCC = oral squamous cell carcinoma.

shown to be associated with HNPCC and some sporadic cases of colon cancer.[5] Indeed, malfunction of the MMR system is associated with decreased genomic stability, which can cause high rates of mutations throughout the genome.[6] Since the first reports of the involvement of the MMR system in colon cancer, several studies have been conducted to evaluate the role of this system in different types of cancer. In OSCC, however, the role of MMR remains unclear. Previous studies have demonstrated that hMLH1 expression is inversely associated with OSCC differentiation[15] and with the degree of dysplasia in oral leukoplakia.[8] Furthermore, hMLH1 and hMSH2 expression decrease throughout the process of lip carcinogenesis.[11] These previous data suggest that MMR deficiency is associated with the progression of oral carcinogenesis and more aggressive tumors. However, the association between these markers and overall survival of OSCC had never been analyzed. Thus, the clinical significance of these events remained unclear. In the present study, patients with higher expression of hMSH6 demonstrated poor survival rates. Moreover, the MutSα complex overexpression was an independent prognostic aspect in the multivariate analysis. Besides MutSα complex, TNM and recurrence were independent prognostic factors of the present study; however, it is important to note that the ratio between sample size and number of events represents a limitation of the present study.

Recently, MMR deficiency has been indicated to be a marker of good prognosis in different solid tumors. Kato et al[23] demonstrated that MMR-deficient endometrial cancer cases had significantly better progression-free and overall survival compared with MMR-retained cases. Moreover, high hMSH6 expression is associated with an increased risk of death from a primary melanoma[16] and, combined with hMSH2 expression, is associated with shorter survival times and presence of metastasis in patients with osteosarcoma.[17] High hMSH6

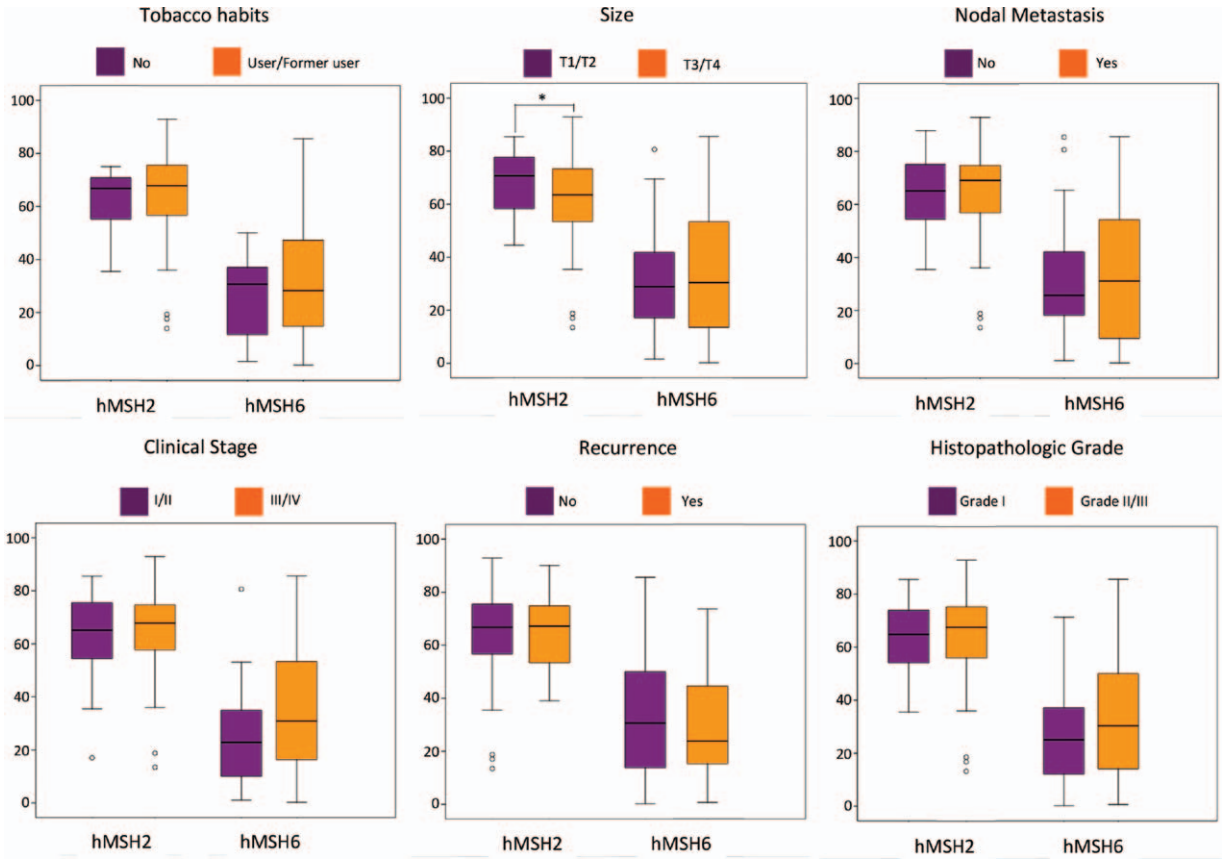

FIGURE 2. Box plots of hMSH2 and hMSH6 according to the main clinical-pathological aspects. Note very homogenous distribution in the expression of both proteins with regard to tobacco habits, nodal metastasis, clinical stage, recurrence, and histopathological grade. In relation to tumor size, patients with higher T status (T3/T4) had lower hMHS2 expression ($P = 0.03$, Mann-Whitney test).

expression has also been associated with a nonresponse to chemotherapy in patients with osteosarcoma[17] and malignant pleural mesothelioma.[18] Therefore, the present results are in agreement with these previous reports and lend support to the notion that the activation of the MutSα complex is associated with more aggressive tumors and can be used as a prognostic marker for different solid tumors, including OSCC.

In the present study, higher hMSH6 expression was associated with poor prognosis, whereas hMSH2 expression was not. Nevertheless, the analysis of both proteins combined (MutSα complex) revealed a significant association between aMutSα complex overexpression with poor prognosis. Furthermore, hMSH2 and hMSH6 were directly correlated, which demonstrates that an increase in hMSH2 is correlated with an increase in hMSH6. The complex formation between hMHS2 and hMSH6 appears to be extremely important. Previous reports have demonstrated that hMSH2 fails to relocate to the nucleus in cells that lack hMSH6.[24] Moreover, both hMSH2 and hMSH6 are required for initiation of the MMR pathway. Thus, analyzing the simultaneous overexpression of both proteins is important to greater precision in the evaluation of MMR activation.

The MutSα complex is regulated by both endogenous stimuli, such as the cell cycle, and exogenous stimuli. hMSH2 levels increase at least 12-fold in proliferating cells in comparison to resting cells.[25] Expression levels of this protein increase during replicative and postreplicative phases of the

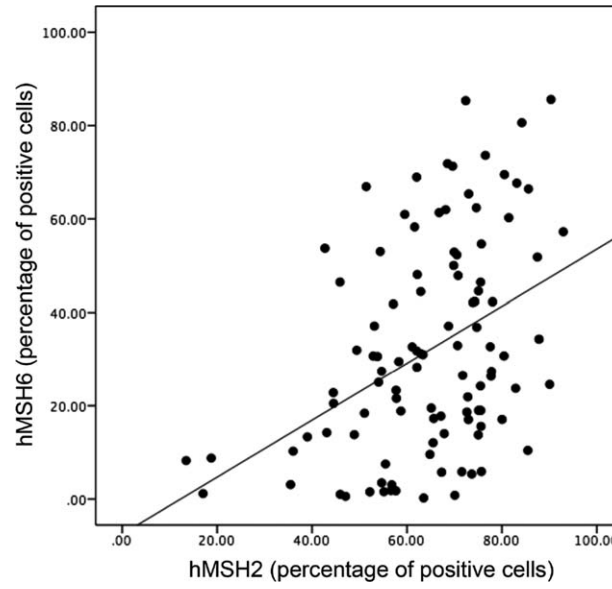

FIGURE 3. Direct correlation between hMSH2 and hMSH6 (scatter plot). Note that an increase in hMSH2 was correlated with an increase in hMSH6 (Spearman correlation coefficient: 0.38; $P < 0.05$).

TABLE 2. Association Between hMSH2 and hMSH6 Expression and Clinical-Pathological Aspects

	hMSH2	*P*	hMSH6	*P*
Tobacco habits				
No	62.75 (±12.04)	0.39	25.85 (±17.06)	0.56
User/former user	64.99 (±15.90)		32.00 (±22.67)	
Size				
T1/T2	68.48 (±10.99)	0.03	29.30 (±18.34)	0.67
T3/T4	61.79 (±16.89)		32.81 (±24.16)	
Nodal metastasis				
Yes	64.44 (±16.99)	0.55	33.18 (±24.36)	0.54
No	64.39 (±12.47)		29.64 (±19.89)	
Clinical Stage				
I/II	63.03 (±15.46)	0.58	24.89 (±18.86)	0.07
III/IV	65.02 (±14.91)		33.86 (±23.12)	
Recurrence				
Yes	64.10 (±12.95)	0.74	28.77 (±20.77)	0.45
No	64.49 (±16.62)		32.77 (±23.72)	
Histopathologic graduation				
Grade I	63.87 (±12.66)	0.56	27.66 (±20.08)	0.35
Grade II/III	64.66 (±16.11)		32.95 (±23.17)	
Evolution				
Life	64.46 (±15.86)	0.79	28.81 (±20.27)	0.24
Dead	64.25 (±14.40)		36.09 (±26.70)	

Mann-Whitney test.

cell cycle. Interestingly, MSH2 levels decrease 4-fold when cells are induced to differentiation.[25] Besides endogenous stimuli, mutagenic treatments, such as alkylating agents, increase the levels of hMSH2 and hMSH6, resulting from the translocation of the complex from the cytoplasm to the nucleus. This inducible response occurs immediately after alkylation and is both long-lasting and dose-dependent.[26] The intracellular location of MMR proteins is crucial, as these proteins need to be in the nucleus to repair DNA.[24] These previous data lend support to the notion that the activation of MutSα complex occurs in undifferentiated cells, with a higher proliferative profile and under mutagenic stimuli. Therefore, these cells must have a more aggressive pattern. This could explain the results of the present study, which demonstrated that MutSα complex overexpression is an independent prognostic factor for poor overall survival in patients with OSCC.

Recently, the development of the Next-Generation Sequencing has significantly improved the discovery of single-base changes, providing a novel perspective for several diseases,[27] including OSCC.[28,29] Among all 106 cases analyzed in both studies cited, only 1 case exhibited hMSH6 mutation.[28] Mutations in other genes involved in DNA repair, such as MYH1, LIG3, BRCA2, and POLD1, have been identified in some cases.[28] From this data, one may presume that the MMR system is not mutated and is functional in OSCC. Moreover, functional inactivation of MMR genes is associated with a lack of protein expression.[7] Therefore, the present findings, which demonstrated that all cases of OSCC in the cohort analyzed exhibited the immunohistochemical expression of hMSH2 and hMSH6, corroborate the assumption that the MMR system is functional in OSCC. The present results also demonstrate that the cases with greater expression of MutSα have poor survival rates.

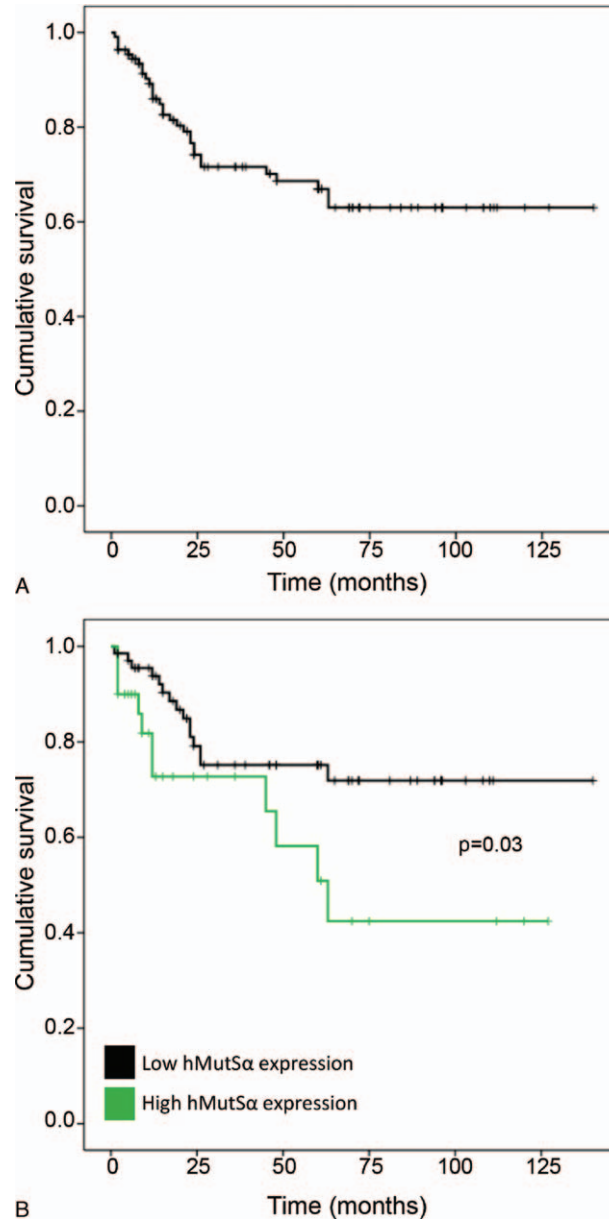

FIGURE 4. Kaplan-Meier analysis of (A) OSCC patient overall survival and (B) according to MutSα expression. Patients with higher expression of MutSα presented more pronounced decline in the survival curve compared with patients with lower MutSα expression in the first year after diagnosis (12-months) and also in a later period (after 50-months) (Log rank test, $P = 0.03$). OSCC = oral squamous cell carcinoma.

This higher expression may indicate an increased activation of this pathway induced by genomic instability. We assume that the MMR pathway in OSCC is not sufficient to induce cell death, presumably due to the lack of function of key genes, such as p53, which is known to be highly mutated in OSCC.

Although future studies are necessary to clarify the role of the MMR system in OSCC, the MutSα complex may constitute a molecular marker for poor prognosis in patients with OSCC. The cutoff values for hMSH2 and hMSH6 proposed in the present study need to be validated in larger studies.

TABLE 3. Predictors of Overall Survival of Patients With OSCC

	Univariable Analysis		Multivariable Analysis	
	HR (95% CI)	***P***	**HR (95% CI)**	***P***
Age	0.97 (0.94–1.00)	0.08		
Gender				
Male	1	0.43		
Female	0.62 (0.18–2.05)			
Tobacco Habits				
No	1	0.16		
User/former user	4.20 (0.56–31.19)			
Pack/years	1.01 (0.99–1.03)	0.17		
Pain				
No	1	0.01		
Yes	6.13 (1.44–26.08)			
Size				
T1/T2	1	0.003		
T3/T4	5.99 (1.80–19.93)			
Nodal metastasis				
Absent	1	0.67		
Present	1.17 (0.55–2.47)			
TNM				
I/II	1	0.003	1	0.01
III/IV	9.13 (2.16–38.56)		4.97 (1.45–16.98)	
Recurrence				
No	1	0.02	1	0.04
Yes	2.33 (1.14–4.76)		2.50 (1.03–6.08)	
Histopathologic graduation				
Grade I	1	0.02		
Grade II/III	3.30 (1–5.07)			
hMSH2	1.01 (0.98–1.03)	0.41		
hMSH6	1.01 (1.00–1.03)	0.03		
MutSα expression				
Low	1	0.03	1	0.02
High	2.27 (1.04–4.97)		2.75 (1.13–6.65)	

CI = confidence interval, HR = hazard ratio. HR and CI estimated by Cox proportional hazard regression model.

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