Reduced expression of mutS homolog 2 and mutL homolog 1 affects overall survival in laryngeal squamous cell carcinoma patients: Investigation into a potential cause

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Abstract. The risk factors affecting the survival rates of laryngeal carcinoma are not well understood. In this study, we investigated the expression status of mutS homolog 2 (MSH2) and mutL homolog 1 (MLH1) and examined the relationship between these two molecules and overall survival rates in laryngeal cancer. We also explored the potential reason for the altered expression of these two genes. Using real-time polymerase chain reaction and western blotting, we detected MSH2 and MLH1 expression in laryngeal cancer tissue samples. We collected a retrospective cohort with 180 laryngeal cancer patients, and inspected MSH2 and MLH1 staining with tissue microarray immunohistochemistry. Prognostic value of clinicopathological characteristics was evaluated by statistical analysis. Laryngeal carcinoma cells were co-cultured with *Helicobacter pylori* (*H. pylori*) bacteria. MSH2 and MLH1 were expressed at lower levels compared to those of adjacent tissues in 21 laryngeal carcinoma patients. Patients with negative expression of MSH2 and MLH1 tended to have a higher risk of mortality compared to patients with positive expression (HR=4.38; HR=3.0, respectively). Cigarette smoking rate was higher in the MLH1 expression positive group. *H. pylori* infection reduced the MSH2 and MLH1 expression levels of laryngeal carcinoma cell lines within co-culture conditions. It is suggested that the altered expression levels of MSH2 and MLH1 probably affect the overall survival of laryngeal carcinoma patients. *H. pylori* infection may have an effect on the expression of MSH2 and MLH1 in laryngeal carcinoma patients.

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Introduction

Head and neck carcinoma is more than twice as common in men than in women; it is the sixth most common cancer in the world, with 640,000 cases of head and neck cancer leading to approximately 350,000 deaths per year. There are approximately 160,000 new cases of laryngeal carcinoma every year (1). To date, the treatment options for laryngeal cancer include surgery, radiotherapy and chemotherapy, either individually or in combination. Treatment options depend on the site and stage of the cancer as well as the overall health status of the patient (2-4). The overall 5-year relative survival rate for this cancer is only 64.2% worldwide (3).

Cigarette smoking and alcohol consumption are factors that have been shown to reduce survival rates of head and neck cancer patients (1,5). Recently, human papillomavirus (HPV) infection, especially HPV-16, was recognized as a risk factor for head and neck cancer (6). This particular virus is considered a prognostic marker for enhanced overall and disease-free survival (6,7). The roles of microorganisms present in the microenvironment of the human body and their contribution to health have been of growing concern in recent years (8). In our previous study, we detected *Helicobacter pylori* (*H. pylori*) in the mucosa of larynx and found that this microbe is likely an independent risk factor for laryngeal squamous cell carcinoma (LSCC). The laryngeal mucosa may provide a reservoir for bacteria and may be a potential staging place for its transmission to other areas (9).

MutS homolog 2 (MSH2) and mutL homolog 1 (MLH1) are the main members of the DNA mismatch repair (MMR) system, which is essential for genome stability and recombination of chromosomes (10). Loss of MMR activity, especially when there is reduced expression of MSH2 and/or MLH1, causes cells to be less sensitive to DNA-damaging agents.

This decreased expression is responsible for promoting carcinogenesis by accelerating the accumulation of mutations in oncogenes and tumor suppressor genes (11). Dysfunction of MSH2 or MLH1 may cause tumors in several organs, such as hereditary non-polyposis colorectal carcinoma and head and neck squamous cell carcinoma (HNSCC) (12,13). With respect to respiratory lung squamous cell carcinoma and oral cancer, MSH2 and MLH1 gene expression levels have been found to be correlated with smoking status. Their expression increase with smoking, and this is associated with the repair of gene damage likely due to chemicals found in tobacco smoke (14,15).

To date, a few research groups have studied the expression patterns of MSH2 and MLH1 in HNSCC (13,14,16-21). Research findings on MSH2 and MLH1 in head and neck cancer come from small groups of clinical samples; the relationship between overall survival rate and MSH2 and MLH1 expression status remains uncertain. In the current study, we investigated the expression of MSH2 and MLH1 in tissue samples from LSCC patients and analyzed the relationship between MSH2, MLH1 and overall survival. We also evaluated the relationship between the expression status of both genes and cigarette smoking, and investigated whether these gene expression levels were altered by *H. pylori* infection.

Materials and methods

LSCC patients. In the present study, two groups (n=201) of LSCC patients from the Eye, Ear, Nose and Throat Hospital, Fudan University, were enrolled. For the first group (n=21), we collected samples from February 2012 to May 2012 for gene expression analysis by real-time polymerase chain reaction (RT-PCR) and western blotting. We also retrospectively collected the second group (n=180) of LSCC subjects from January 2006 to May 2012. The clinicopathological data and clinical specimens from the tumors were used for molecular and statistical analyses. All tumor specimens were confirmed to be squamous cell carcinoma by the Department of Histopathology and patients were treated by total or partial laryngeal resection in our hospital. Exclusion criteria were: i) preoperative anticancer chemotherapy and/or radiotherapy; ii) diagnosis with additional cancer; iii) postoperative radiotherapy and/or chemotherapy; and iv) refusal of consent. Tumor stage was determined according to the International Union Against Cancer TNM classification system, 6th edition (22). Tumor volume measurement was previously described (23). All enrolled subjects provided written informed consent in accordance with the committee's regulations. This study was approved by the Human Research Ethics Committee at the Eye, Ear, Nose and Throat Hospital, Fudan University. Follow-up information for all participants was updated every four months. If recurrence was not diagnosed, patients were censored on the date of mortality or last follow-up. Overall survival was defined as the interval time between the dates of surgery and mortality.

Tissue microarray (TMA). To detect MSH2 and MLH1 gene expression patterns, TMA sets were constructed from 180 archived paraffin-embedded pretreatment LSCC biopsies in the Department of Pathology (Shanghai Biochip Co., Ltd., Shanghai, China). Briefly, screening of hematoxylin-eosin-stained slides for optimal tumor tissue was carried out by an experienced pathologist. Two paired core punches were obtained from formalin-fixed and paraffin-embedded LSCC samples by using punch cores and placing them into TMA blocks. Consecutive 4-mm sections were placed on 3-aminopropyltriethoxysilane-coated slides. Unstained 4.0 mm sections from these blocks were used for MSH2 and MLH1 staining (24).

Immunohistochemistry. The primary monoclonal antibodies used for MSH2 (ab52266) and MLH1 (ab92312) (both from Abcam Co., USA) and the protocols followed have been described elsewhere (24). Immunohistochemical labeling was assessed on a compound microscope by two of the authors who had no knowledge of the patient clinical characteristics.

Evaluation of immunohistochemistry. The method of evaluation of immunohistochemistry findings has previously been described (24,25). The expression of MSH2 and MLH1 was measured with a computerized imaging system, composed of a Leica CCD camera (DFC420) connected to a Leica DM IRE2 microscope (Leica Microsystems Imaging Solutions Ltd.). MSH2 and MLH1 density was measured by Image-Pro software (Media Cybernetics, Inc., Bethesda, MD, USA). To read each antibody staining, a uniform setting was used for all the slides. Optical density of all the positive staining of MSH2 and MLH1 in each image was measured and its ratio to total area of each image was calculated to determine MSH2 and MLH1 density. The Leica software measured MSH2 and MLH1 positive areas in the image (25).

Bacterial strain infection and cell lines. *H. pylori* strain 11637 was kindly donated by the Shanghai Institute of Digestive Diseases and Shanghai Renji Hospital. We used the multiplicity of infection (MOI) at a bacteria:cell ratio of 30:1, 100:1 and 300:1. Infected cells were harvested at the 3, 6, 9, 12, 24, 48 and 72 h time-points. The LSCC cell lines HEp-2 and AMC-HN-8 were cultured in RPMI-1640 medium (Invitrogen, Shanghai, China) with 10% fetal bovine serum, 100 units penicillin/ml and 100 μg streptomycin/ml at 37˚C in a humidified incubator with a 5% CO_2 atmosphere.

RNA extraction and reverse transcription-PCR. Tissue samples from 21 LSCC subjects were used to detect MSH2 and MLH1 gene expression. Total RNA was extracted from clinical tissue specimens and culture cells using TRIzol reagent (Invitrogen) and the RNeasy mini kit (Takara Bio, Inc., Dalian, China) respectively, according to the manufacturer's protocols. The total RNA quantity was measured using a NanoDrop-1000 (NanoDrop Technologies, Inc., USA). Total RNA was reverse-transcribed to cDNA according to the manufacturer's protocol.

cDNA quantitation by RT-PCR. RT-PCR was performed to evaluate the expression levels of the MSH2 and MLH1 genes. Two sets of primers were used: forward, 5'-gtc agc ttc cat tgg tgt tgt-3' and reverse, 5'-cat gtc tcc agc agt ctc tcc-3'; forward, 5'-ggc cat tgt cac aga gga taa-3' and reverse, 5'-ggg aat cat ctt cca cca ttt-3', for MSH2 and MLH1, respectively. RT-PCR was performed according to the manufacturer's protocol (Takara Bio, Inc.).

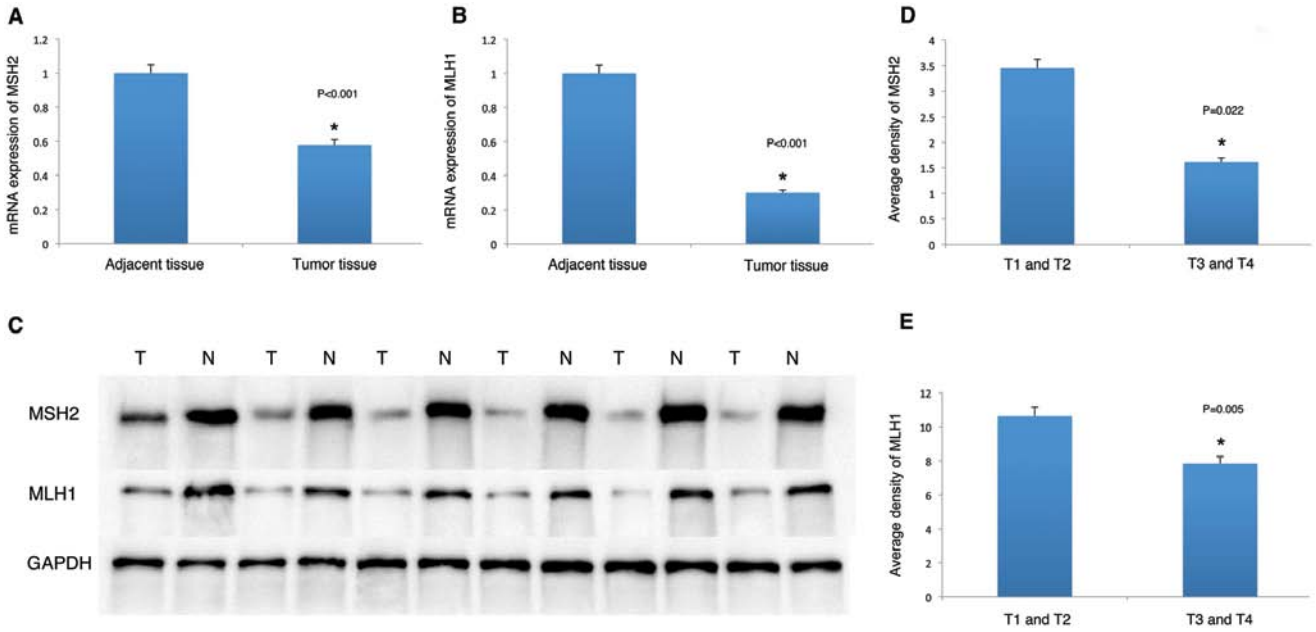

Figure 1. mRNA and protein expression of mutS homolog 2 (MSH2) and mutL homolog 1 (MLH1) in laryngeal squamous cell carcinoma (LSCC) patient samples. (A and B) mRNA expression levels of MSH2 and MLH1 in tumor and corresponding adjacent normal tissues. (C) The MSH2 and MLH1 protein expression status in tumor (T) and adjacent normal (N) tissue from five LSCC subjects. (D and E) Average densities of MSH2 and MLH1 in T1,T2 and T3,T4 stages. *Independent two-sample t-test showed a statistical difference.

Western blotting. Western blotting was used to detect the protein levels of both MSH2 and MLH1. The primary antibodies used in this study were ab52266 and ab92312 (Abcam Co.).

Statistical analysis. Statistical analysis was performed using SPSS 13.0. Pearson Chi-square, Mann-Whitney and Fisher's exact tests were used to analyze the clinicopathological parameters. Kaplan-Meier analysis was used to determine the overall survival rate. Log-rank test was used to compare patient survival between subgroups. The Cox regression model was used to perform multivariate analysis. For *H. pylori* infection, results are expressed as means ± SEM. Comparisons were made with the Student's t-test and differences were considered statistically significant if $P<0.05$. All P-values were determined from two-sided tests.

Results

MSH2 and MLH1 mRNA and protein expression levels of 21 patients. MSH2 and MLH1 mRNA was isolated from 21 LSCC patient tumors and adjacent normal tissues, and tested by RT-PCR. It was found that MSH2 and MLH1 in tumor tissues had lower expression levels than in adjacent normal tissues ($P<0.001$ for both genes) (Fig. 1A and B). The levels of MSH2 and MLH1 proteins were also detected by western blotting. There was a statistically significant difference between tumors and adjacent normal tissues. These two proteins were both notably lower in tumor tissues compared to adjacent normal tissues in 9/21 LSCC patients (42.9%). Two subjects showed reduced MSH2 expression with MLH1 expression levels equal to adjacent normal tissues (Fig. 1C).

MSH2 and MLH1 immunohistochemistry results. MSH2 and MLH1 staining in 180 tumor tissues was detected by immunohistochemistry in TMA. For MSH2 expression status, 18 LSCC subjects were positive (18/180). For MLH1 expression, 85 subjects were positive (85/180) (Table I). The average densities of MSH2 and MLH1 in stages T1 and T2 were significantly higher than those of stages T3 and T4 ($P=0.022$ and $P=0.005$, respectively). A gradient of MSH2 and MLH1 distribution was observed with T stages, with the density of MSH2 and MLH1 decreasing as the T stage of the tumor increased (Figs. 1D and E and 2).

Cigarette smoking status was higher in the MLH1 positive expression group of samples ($P=0.02$) (Table I).

Retrospective cohort study of 180 LSCC patients. The 3- and 5-year overall survival rates were 90.7 and 86.7% in the MSH2-positive group, 59.6 and 47.3% in the MSH2-negative group, 82.9 and 74.0% in the MLH1-positive group, and 72.1 and 27.2% in the MLH1-negative group. Variables of the retrospective cohort were comprehensively analyzed. The median follow-up time was 35 months (range, 4-81 months). Among the 180 LSCC patients, 104 (57.78%) subjects were alive and 76 (42.22%) subjects had died at the time of analysis. MSH2 and MLH1 expression levels in LSCC subjects were analyzed for correlation with overall survival using Kaplan-Meier analysis and log-rank test for significance estimation. For MSH2 statistical analysis, patients were divided into two groups, those with positive and those with negative immunostaining. A statistically significant difference in cumulative overall survival was found between LSCC patients with MSH2 positive staining and negative staining. Kaplan-Meier survival curves indicated that the MSH2-negative LSCC patients correlated with poorer overall survival, with HR being 6.27 (95% CI, 1.53-25.62; $P=0.01$) (Table II). For evaluation of MLH1 staining, LSCC subjects were divided into two groups, those positive and those with negative for MLH1 expression. Our results showed that

Table I. Clinicopathological characteristics of the retrospective cohort of 180 LSCC patients in the current study.

	MLH1 positive N (%)	MLH1 negative N (%)	P-value	MSH2 positive N (%)	MSH2 negative N (%)	P-value
Gender			0.55[a]			0.41[c]
Female	2 (2.4)	3 (3.2)		1 (5.6)	4 (2.5)	
Male	83 (97.6)	92 (96.8)		17 (94.6)	158 (97.5)	
Age			0.25[a]			0.92[a]
<60	33 (38.8)	45 (47.7)		8 (44.4)	70 (43.2)	
≥60	52 (61.2)	50 (52.6)		10 (55.6)	92 (56.8)	
T stage			0.24[b]			0.44[b]
T1	16 (18.8)	14 (14.7)		5 (27.8)	25 (15.4)	
T2	37 (43.5)	38 (40.0)		6 (33.3)	69 (42.6)	
T3	27 (31.8)	34 (35.8)		6 (33.3)	55 (34.0)	
T4	5 (5.9)	9 (9.5)		1 (5.6)	13 (8.0)	
Clinical stage			0.11[b]			0.37[b]
I	16 (18.8)	14 (14.7)		4 (22.2)	26 (16.0)	
II	33 (38.8)	29 (30.5)		6 (33.3)	56 (34.6)	
III	20 (23.5)	27 (28.4)		6 (33.3)	41 (25.3)	
IV	16 (18.8)	25 (26.3)		2 (11.1)	39 (24.1)	
Tumor location			0.95[b]			0.34[b]
Supraglottic	28 (32.9)	33 (34.7)		4 (22.2)	57 (35.2)	
Glottic	56 (65.9)	59 (62.1)		14 (77.8)	101 (62.3)	
Subglottic	1 (1.2)	3 (3.2)		0 (0)	4 (2.5)	
Tumor differentiation			0.22[a]			0.72[a]
>II	36 (42.4)	31 (32.6)		6 (33.3)	61 (37.7)	
≤II	49 (57.6)	64 (67.4)		12 (66.7)	101 (62.3)	
Tumor size			0.65[b]			0.32[b]
≤1 cm^3	31 (36.5)	29 (30.5)		7 (38.9)	53 (32.7)	
1-10 cm^3	34 (40.0)	45 (47.4)		9 (50.5)	70 (43.2)	
≥10 cm^3	20 (23.5)	21 (22.1)		2 (11.1)	39 (24.1)	
Drinking status			0.49[b]			0.054[b]
Non drinking	19 (22.4)	28 (29.5)		1 (5.6)	45 (27.8)	
Drinking						
≤36 g/day	23 (27.1)	22 (22.2)		5 (27.8)	40 (24.7)	
>36 g/day	43 (50.6)	45 (47.4)		12 (66.7)	77 (47.5)	
Smoking status			0.02[a]			1[c]
Non smoking	7 (8.2)	20 (21.1)		2 (11.1)	25 (15.4)	
Smoking	78 (91.8)	75 (78.9)		16 (88.9)	137 (84.6)	

[a]P-value was tested from Pearson Chi-square test; [b]P-value was tested from Mann-Whitney test. [c]P-value was tested from Exact test. LSCC, laryngeal squamous cell carcinoma; MLH1, mutL homolog 1; MSH2, mutS homolog 2.

subjects negative for MLH1 had a higher risk of mortality than subjects with positive staining (HR=3.87; 95% CI, 2.29-6.56). The Kaplan-Meier survival curves of MSH2 and MLH1 are presented in Fig. 3.

A Cox regression analysis model was applied to adjust confounding factors with gender, age, T stage, N stage, clinical stage, smoking, drinking, tumor size, tumor location, and tumor differentiation status. After multivariate analysis, it was observed that patients with negative expression of MSH2 and MLH1 tended to have a higher risk of mortality than patients with positive expression (HR=4.38; 95% CI, 1.05-18.25; P=0.04; HR=3.0, 95% CI, 1.76-5.14; P<0.001, respectively) (Table II and Fig. 3).

Investigation of the potential contribution of altered MSH2 and MLH1 expression in LSCC. Our previous research study observed that *H.pylori* was present in the laryngeal mucosa and was an independent risk factor for LSCC (9). We co-cultured

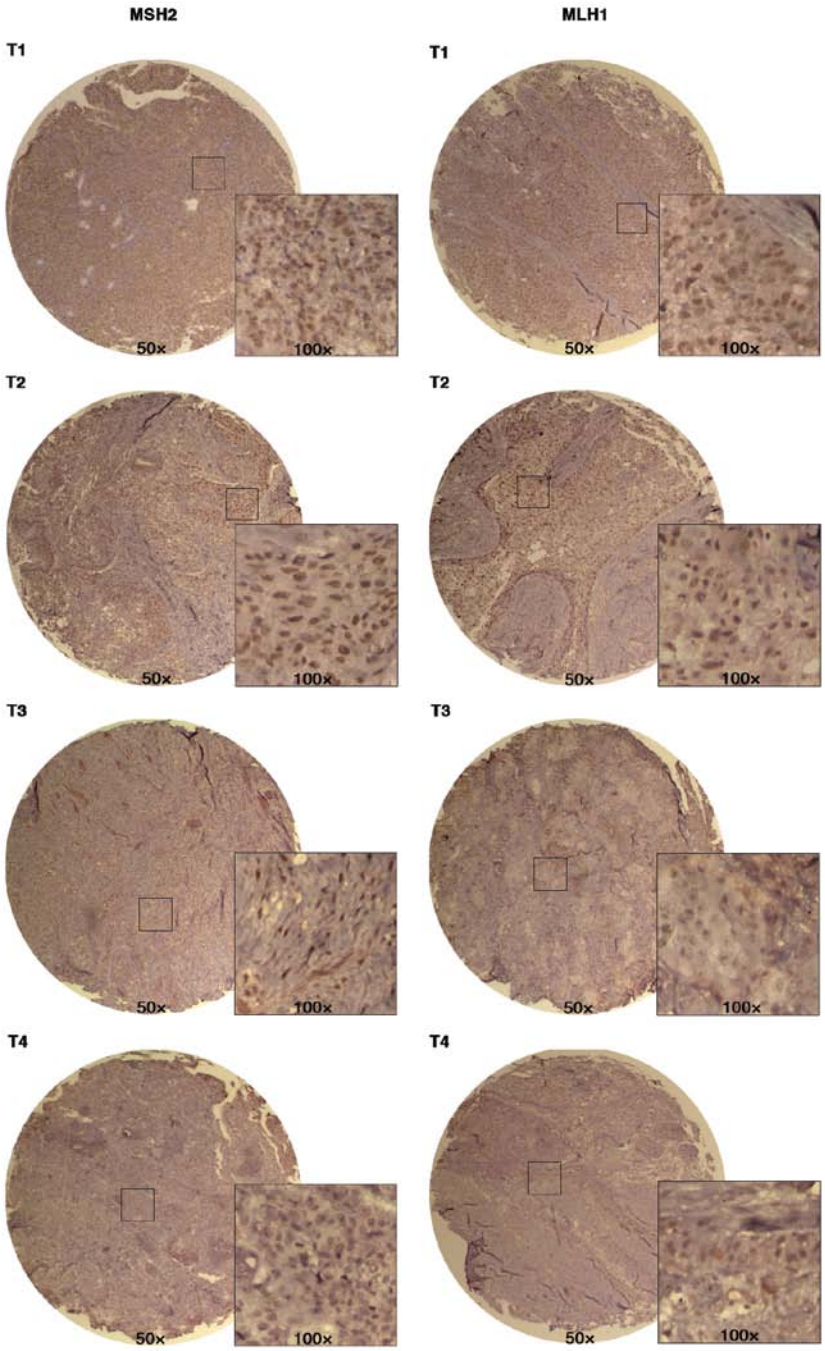

Figure 2. Images of immunostaining of mutS homolog 2 (MSH2) and mutL homolog 1 (MLH1). The left lane shows the MSH2 immunohistochemical microarray blocks, which were from T1 to T4. The right lane shows the MLH1 blocks, which were from T1 to T4. Magnification, x50 and x100.

HEp-2 cells and the *H. pylori* wild-type strain 11637 and detected the mRNA expression levels of MSH2 and MLH1 at 3, 6, 9, 12, 24, 48 and 72 h, and at the MOIs of 30:1, 100:1 and 300:1. We found that the mRNA levels of MSH2 and MLH1 were reduced and the magnitude of the reduction was increasingly significant with an increasing infection time, when compared with uninfected control cells. We also co-cultured AMC-HN-8 cells with strain 11637 at different times and MOIs and obtained the same results. Western blotting was used to confirm this finding and showed that MSH2 and MLH1 levels were altered by *H. pylori* infection at the MOI of 100:1 (Fig. 4).

Discussion

In the present study, we found that MSH2 and MLH1 mRNAs and proteins have lower expression in LSCC tumors than in adjacent normal tissues. The negative immunostaining of these two proteins tended to predict poorer survival status compared to patients with positive expression. MLH1 gene expression levels were correlated with cigarette smoking status. *H. pylori* may be a potential bacterial pathogen that affects the expression status of both MSH2 and MLH1 in LSCC patients.

MSH2 and MLH1 expression levels have been found to provide beneficial prognostic information and staining status

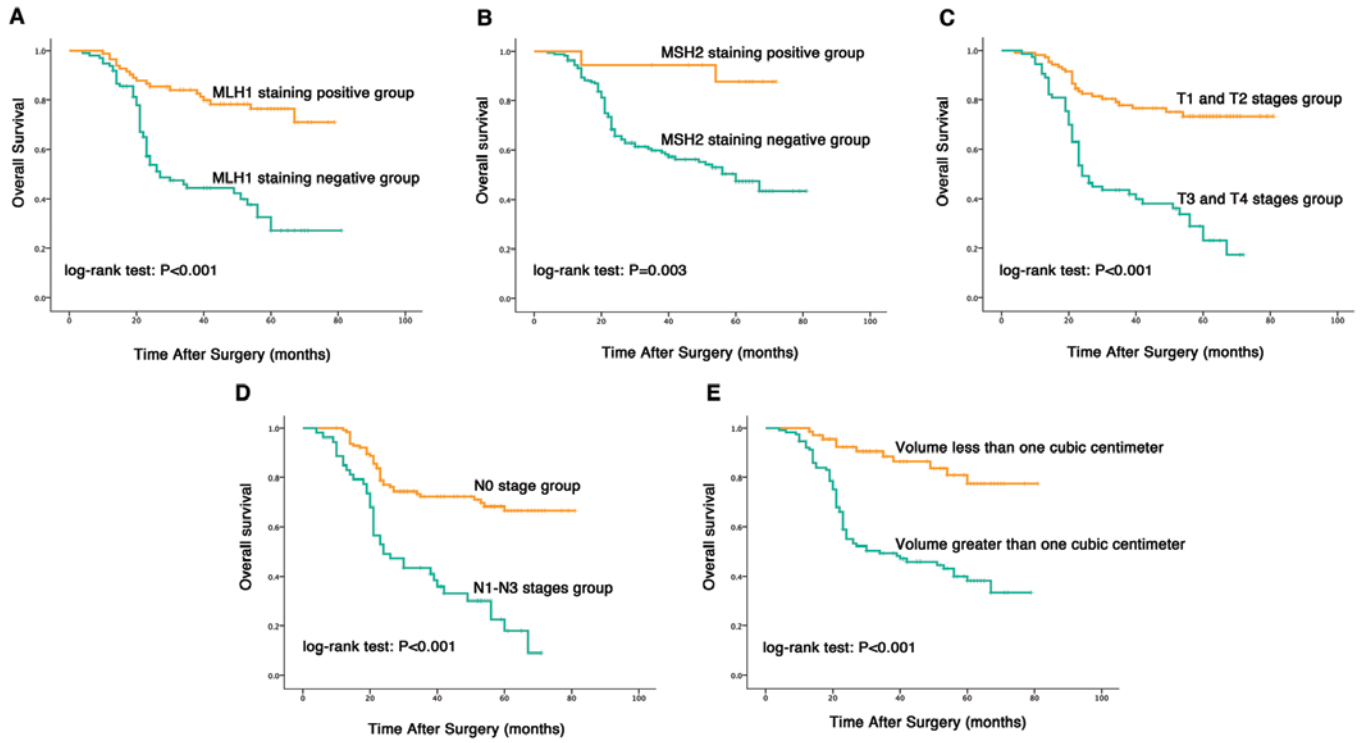

Figure 3. Kaplan-Meier survival curves of laryngeal squamous cell carcinoma (LSCC) patients with clinical characteristics. (A and B) MutS homolog 2 (MSH2) and mutL homolog 1 (MLH1) expression positive and negative LSCC subjects were associated with cumulative overall survival. (C) T3 and T4 patients had a lower survival rate than T1 and T2 stage LSCC subjects. (D) Survival curves for patients with N0 and N1-N3 stages. (E) Correlation of tumor volume less than or equal to one cubic centimeter and volume greater than one cubic centimeter with overall survival curve.

is taken into consideration when making tailored treatments, particularly in colorectal cancer (12). Reduction of the expression levels of MSH2 and MLH1 may play a significant role in the development of chemotherapy resistance (16). Additionally, these two genes are involved in synthetic lethal approaches to cancer treatment such as PINK1, which represents a potential therapeutic target involving MSH2-and MLH1-related MMR deficiency (11).

Deficiency in MMR genes is not only associated with colorectal carcinoma. Indeed, other types of human cancer may also be affected, such as HNSCC (13). To date, the mechanism of MSH2 and MLH1 in HNSCC remains uncertain. Some studies have reported that MMR gene expression levels were low in HNSCC patients and suggested that this low expression of MLH1 was associated with increased risk of head and neck carcinoma (13,20). Caldeira *et al* (17) suggested that MLH1 expression is reduced in oral leukoplakias and this alteration is an early event in oral carcinogenesis. In addition, MMR deficiency has been linked to resistance to chemotherapeutic approaches and methylation agents. MLH1 expression is a predictor of cisplatin sensitivity and also predicts the sensitivity of HNSCC to platinum-based chemotherapy (16). Hypermethylation of MSH2 and MLH1 may play a role in oral carcinogenesis and may be correlated with a tendency to develop multiple oral malignancies (18). However, this is rather controversial as another study reported opposite findings. Marani *et al* (19) found that MSH2 and MLH1 are upregulated in three of six mucosal melanomas in head and neck patients. Wang *et al* (21) reported that the expression of MSH2 and MLH1 was not involved in HNSCC, indicating that inactivation of these two genes does not play a role in the development of microsatellite instability in tumors in this patient group.

Therefore, it is imperative to verify the relationship between MSH2 and MLH1 expression levels and head and neck carcinoma. We studied two groups of subjects and analyzed the expression patterns of the two genes and patient clinicopathological characteristics. We observed that MSH2 and MLH1 had a lower expression level in tumor tissues than in adjacent normal tissues. Immunohistochemistry was used to detect the expression status of MSH2 and MLH1 in the retrospective cohort group specimens, since this experimental approach has a high sensitivity and specificity for MMR proteins (26). The distribution of the two proteins could be observed in T1 to T4 stages, with the average density of MSH2 and MLH1 decreasing as the T stages of the tumor increased. MMR deficiency continues to be one of the most consistent and promising molecular predictors of prognosis for colorectal cancer and provides strong prognostic information (12). In our univariate analysis, we discovered that the negative expression of MSH2 and MLH1 was correlated with poor overall survival. This observation was verified after adjustment of confounding factors with a Cox regression model.

It has been reported that MSH2 and MLH1 expression is directly influenced by tobacco use (14,15). The increased expression is related to cigarette smoking, suggesting that the biology of MLH1 may be varied by the destructive stimuli (14). In our study, we observed that cigarette-smoking status was higher in LSCC patients with positive MLH1 expression. Thus, this observation supports the previous conclusion.

Table II. Univariate and multivariate Cox regression analyses of risk of mortality in MSH2 and MLH1 gene expression positive and negative patients.

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Gender		0.76	NA	NA
Female	1 (reference)			
Male	0.80 (0.20-3.27)			
Age		0.44	NA	NA
<60	1 (reference)			
≥60	0.84 (0.53-1.32)			
T stage		<0.001		0.03
T1 and T2	1 (reference)		1 (reference)	
T3 and T4	3.97 (2.45-6.42)		1.90 (1.07-3.38)	
N		<0.001		0.03
N0	1 (reference)		1 (reference)	
N1-N3	3.48 (2.21-5.49)		1.74 (1.06-2.85)	
Clinical stage		<0.001	NS	NS
I-II	1 (reference)			
III-IV	1.25 (1.14-1.38)			
Tumor differentiation		0.002	NS	NS
>II	1 (reference)			
≤II	2.35 (1.38-3.99)			
Tumor location		0.003	NS	NS
Glottic	1 (reference)			
Supraglottic and subglottic	1.40 (1.12-1.76)			
Tumor size		<0.001		0.004
≤1 cm^3	1 (reference)		1 (reference)	
>1 cm^3	4.56 (2.40-8.65)		2.88 (1.40-5.94)	
Smoking		0.55	NA	NA
No smoking	1 (reference)			
Smoking				
≤20/day	1.02 (0.54-1.92)	0.95		
>20/day	0.73 (0.40-1.32)	0.29		
Drinking		0.45	NA	NA
No drinking or ≤36 g/day	1 (reference)			
>36 g/day	1.19 (0.76-1.87)			
MSH2 expression		0.01		0.04
Positive	1 (reference)		1 (reference)	
Negative	6.27 (1.53-25.62)		4.38 (1.05-18.25)	
MLH1 expression		<0.001		<0.001
Positive	1 (reference)		1 (reference)	
Negative	3.87 (2.29-6.56)		3.0 (1.76-5.14)	

NA, not adopted; NS, not significant; MSH2, mutS homolog 2; MLH1, mutL homolog 1.

Reasons for reduced expression of MSH2 and MLH1 have yet to be clearly addressed. Potential causes include methylation, tobacco, and local inflammation. These may also be the reasons for initiation and development of laryngeal carcinoma (10). Our previous study reported that *H. pylori* is present in the mucosa of the larynx. This microorganism is also an independent risk factor for LSCC. The laryngeal mucosa thus provides a reservoir for the bacteria and is a likely staging place for its transmission to other areas (9). This finding has also been verified by other studies (27-29). To

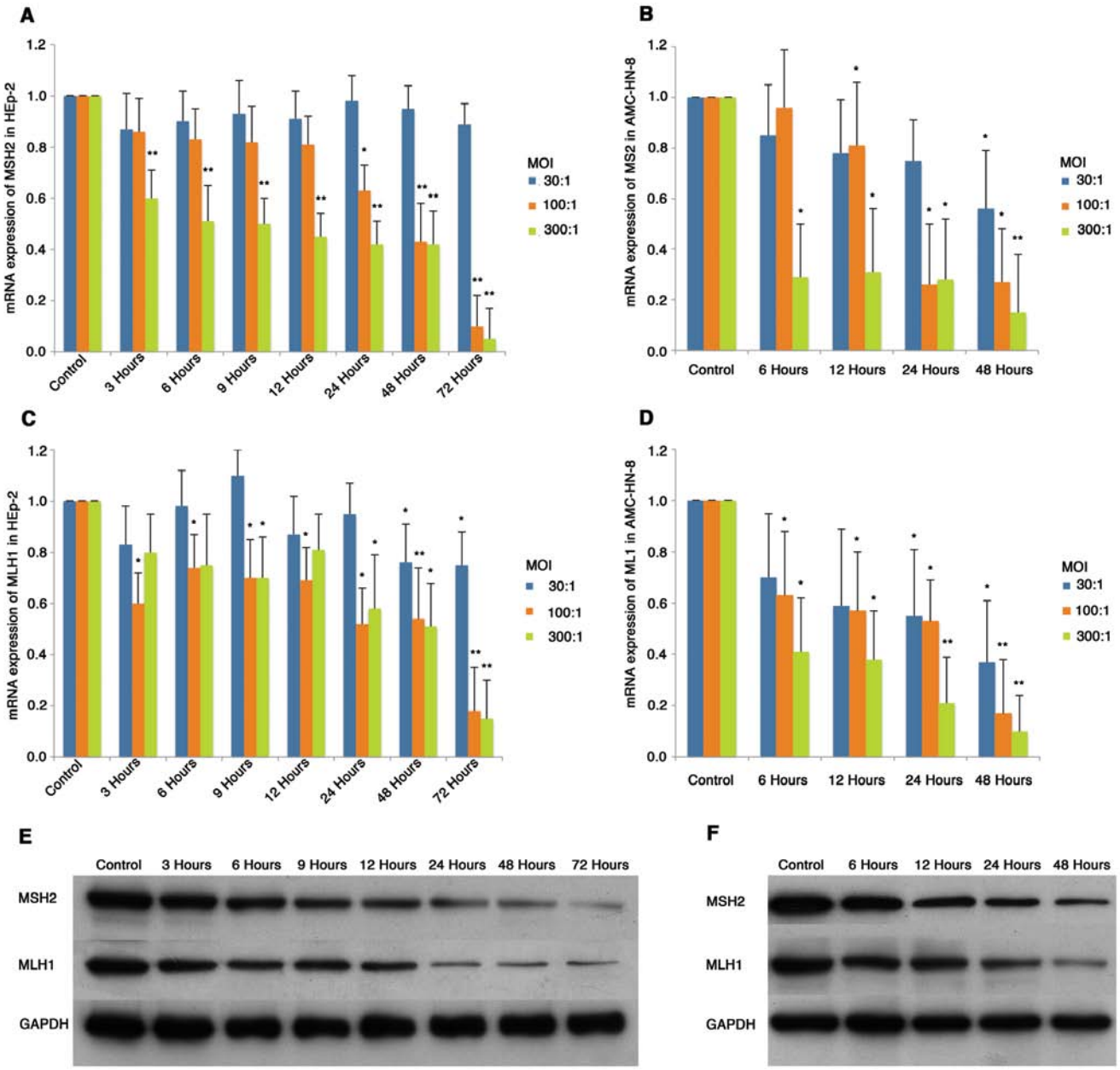

Figure 4. Characterization of mutS homolog 2 (MSH2) and mutL homolog 1 (MLH1) expression levels in laryngeal squamous cell carcinoma (LSCC) cells infected by *H. pylori*. (A and C) MSH2 and MLH1 presented a decreased expression induced by *H. pylori* in the HEp-2 cell line from 3 to 72 h and at multiplicities of infection (MOI) of 30, 100 and 300. (B and D) Reduced expression of MSH2 and MLH1 induced by *H. pylori* infection in the AMC-HN-8 cell line from 6 to 48 h and at MOI of 30, 100 and 300. (E and F) Protein expression in HEp-2 cells infected for 3 to 72 h and AMC-HN-8 cells from 6 to 48 h at MOI of 100. Data are expressed as means ± SEM. *P<0.05; **P<0.001. N=3.

elucidate its potential role in laryngeal cancer, we investigated whether *H. pylori* alters the expression of MSH2 and MLH1. We constructed an *H. pylori* infection model to test this hypothesis and found that the MSH2 and MLH1 expression levels were reduced. Moreover, the magnitude of the reduction was increasingly significant as infection time increased and MOI was enhanced, compared with uninfected controls. These results suggest that *H. pylori* colonizes the mucosa of the larynx and that this infection may affect the expression of MSH2 and MLH1 in LSCC patients. *H. pylori* may act as a carcinogen in laryngeal cancer. However, this novel conclusion requires further investigation and therefore future studies are warranted.

In conclusion, the present study demonstrated that the expression of MSH2 and MLH1 was lower in tumors than in adjacent normal tissues in LSCC patients. This altered expression of MSH2 and MLH1 likely affects the overall survival of laryngeal carcinoma patients. We also investigated a potential reason for this alteration, and found that *H. pylori* infection in the laryngeal mucosa may affect the expression of MSH2 and MLH1.

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