Prognostic value of *MLH1* promoter methylation in male patients with esophageal squamous cell carcinoma

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Abstract. The DNA mismatch repair (MMR) gene MutL homolog 1 (*MLH1*) is critical for the maintenance of genomic integrity. Methylation of the *MLH1* gene promoter was identified as a prognostic marker for numerous types of cancer including glioblastoma, colorectal, ovarian and gastric cancer. The present study aimed to determine whether *MLH1* promoter methylation was associated with survival in male patients with esophageal squamous cell carcinoma (ESCC). Formalin-fixed, paraffin-embedded ESCC tissues were collected from 87 male patients. *MLH1* promoter methylation was assessed using the methylation-specific polymerase chain reaction approach. Kaplan-Meier survival curves and log-rank tests were used to evaluate the association between *MLH1* promoter methylation and overall survival (OS) in patients with ESCC. Cox regression analysis was used to obtain crude and multivariate hazard ratios (HR), and 95% confidence intervals (CI). The present study revealed that *MLH1* promoter methylation was observed in 53/87 (60.9%) of male patients with ESCC. Kaplan-Meier survival analysis demonstrated that *MLH1* promoter hypermethylation was significantly associated with poorer prognosis in patients with ESCC (P=0.048). Multivariate survival analysis revealed that *MLH1* promoter hypermethylation was an independent predictor of poor OS in male patients with ESCC (HR=1.716; 95% CI=1.008-2.921). Therefore, *MLH1* promoter hypermethylation may be a predictor of prognosis in male patients with ESCC.

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Introduction

Esophageal cancer (EC) is a lethal disease that presents a global health threat to humans (1) particularly in developing countries, including China (2). A major histological subtype of EC is esophageal squamous cell carcinoma (ESCC) (3). Despite medical and surgical advances, the prognosis for EC remains poor (4).

Esophageal cancer may be caused by various environmental factors, including chronic exposure to nitrosamines, obesity, smoking and alcohol (5). Tobacco and alcohol consumption are the predominant risk factors for ESCC (6,7). In addition, accumulating epidemiological studies indicated an association between obesity and EC (2,8-10).

Encompassing the genetic and environmental aspects of cancer development, epigenetic modification is considered to have a crucial role in the carcinogenesis of EC (11). DNA methylation is as a key mechanism in the inhibition of the expression of tumor suppressor genes (12). Aberrantly methylated genes have previously been identified as prognostic markers in EC (13,14).

DNA mismatch repair (MMR) genes, including MutL homolog (*MLH)1*, *MLH3*, MutS protein homolog *(MSH)2* and *MSH3*, are critical for maintaining genomic integrity (15,16). The loss of MMR function is closely correlated with carcinogenesis (17-20). *MLH1* encodes a protein that has been implicated in the maintenance of genome stability during DNA replication (21-23). A previous study indicated that *MLH1* promoter hypermethylation was able to inactivate gene transcription, inducing defects in the function of the DNA repair system (24). Therefore, *MLH1* contributes to the subsequent development of tumors including ESCC (24,25). *MLH1* promoter methylation is significantly higher in EC tissues, as compared with paired adjacent tissues (15); however, the association between *MLH1* promoter methylation and the prognosis of EC has yet to be elucidated.

In the current study, *MLH1* methylation was investigated in a total of 87 male patients with ESCC, with the aim of

determining the prognostic value of *MLH1* methylation in this disease.

Materials and methods

Tissue samples. In a retrospective study, formalin-fixed paraffin-embedded (FFPE) tissues (4 μm in thickness) from the primary tumors of 87 male patients diagnosed with ESCC were selected from the archive at the Department of Thoracic Surgery, Shaoxing People's Hospital (Shaoxing, China) between October 1998 and June 2007. No preoperative radiotherapy or chemotherapy was administered prior to the collection of FFPE samples. The paraffin-embedded tissue block of ESCC was sliced transversely using Leica RM2245 Semi-Automated Rotary Microtome (Leica Microsystems GmbH, Wetzlar, Germany). All sections were reviewed with minimum of 75% malignant cells. Pathological parameters were defined according to the World Health Organization guidelines and Union for International Cancer Control tumor-node-metastasis classifications (26). Follow-up data was recorded for 70/87 patients until January 2010, and the maximum follow up period was 11 years and 3 months. The overall survival (OS) time was evaluated from the date of primary surgery to patient mortality, or to the date of the final follow-up (27). Information on clinicopathological characteristics was retrieved from the patient medical records and pathological diagnosis. The present study was approved by the Bioethics Committee of Shaoxing People's Hospital and written informed consent was obtained from all participants.

DNA isolation and bisulfite conversion. Genomic DNA was extracted from the tissue samples using the E.Z.N.A® Tissue DNA kit (Omega Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer's protocol. DNA concentrations were evaluated using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). Bisulfite conversion of genomic DNA was performed using the EZ DNA Methylation-Gold™ kit (Zymo Research Corp., Irvine, CA, USA), which converted only unmethylated cytosine to uracil.

Methylation-specific polymerase chain reaction (MSP). MSP was performed to qualitatively detect the methylation status of *MLH1* (Fig. 1). The total amplification involved a reaction volume of 20 μl, containing 0.5 μl forward and reverse primers, 1.6 μl sodium bisulfate-modified DNA, 10 μl ZymoTaq™ PreMix (Zymo Research Corp.) and 7.4 μl DNase/RNase-free water. The applied MSP primers were as described previously (28), whereas the primer sequences for the *MLH1* methylated (M) alleles were as follows: Forward, 5'-AACGAATTAATAGGAAGAGCGGATAGCG-3' and reverse, 5'-CGTCCCTCCCTAAAACGACTACCC-3'. The primer sequences for *MLH1* unmethylated (U) alleles were forward, 5'-TAAAAATGAATTAATAGGAAGAGTGGATAGTG-3' and reverse, 5'-AATCTCTTCATCCCTCCCTAAAACA-3'. For PCR, the M and U primer pairs were initially denatured at 95˚C for 10 min, followed by 35 cycles with 30 sec denaturation, 45 sec annealing at 55˚C, and 1 min extension at 72˚C. Following a subsequent 7 min extension at 72˚C, the product was stored at 4˚C. A number of DNA samples were also randomly sequenced to determine a complete bisulfite conversion, using the Applied Biosystems™ 3730 DNA Analyzer (Thermo Fisher Scientific, Inc., Waltham, MA, USA; Fig. 1).

Statistical analysis. Statistical analysis was conducted with SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). The Fisher's exact probability method or χ^2 test was performed to compare the methylation frequencies between the groups. The Kaplan-Meier method was used to estimate the medium OS time for survival analyses. Log-rank tests were used for comparison of the survival curves. Potentially important factors in univariate analyses were then included in multivariate analyses. The Cox proportional hazards models was applied to calculate hazard ratios (HR) and the corresponding 95% confidence intervals (CI). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patient characteristics. As presented in Table I, the median survival time of patients with ESCC was 74.0 months, ranging from 1.5-135 months. The mean age of patients with ESCC was 62.63±8.48 years. Of the 87 patients with ESCC, 34 (39.1%) and 53 (60.9%) cases were in the early-stage (I and II) and the advanced-stage (III and IV), respectively. In addition, 43 (49.4%) and 42 (48.3%) patients consumed alcohol and smoked cigarettes, respectively. Patients were classified as being alcohol consumers if they had consumed at least one alcoholic drink in the last 30 days (29). Smoking was classified based upon whether the patient had smoked continuously for >6 months during their lifetime (30).

MLH1 promoter methylation in ESCC tissues. As presented in Table I, *MLH1* promoter methylation was observed in 53/87 (60.92%) ESCC tissue samples. No significant differences were observed in further correlation analyses between *MLH1* methylation and clinical phenotypes (All $P>0.05$). The evaluated clinical phenotypes were as follows: Age at diagnosis, smoking behavior, history of alcohol consumption and clinicopathological characteristics, including tumor location, histological differentiation, lymph node metastasis, clinical stage, surgical margin status and vascular invasion.

Survival analysis. As presented in Table II, the univariate survival analysis demonstrated that the OS of patients with ESCC was associated with age at the time of surgery ($P=0.028$), history of alcohol consumption ($P=0.015$) and vascular invasion ($P=0.015$). Meanwhile, *MLH1* promoter methylation was significantly associated with poor OS in patients with ESCC ($P=0.048$; Fig. 2). Following adjustments for these potential confounding factors in a multivariate Cox proportional hazards model, the OS of patients with ESCC was observed to be significantly associated with *MLH1* promoter hypermethylation (HR=1.716; 95% CI=1.008-2.921), age ≤60 years old (HR=0.486, 95%; CI=0.279-0.849), alcohol consumption (HR=1.968; 95% CI=1.157-3.348) and vascular invasion (HR=1.791; 95% CI=1.007-3.185).

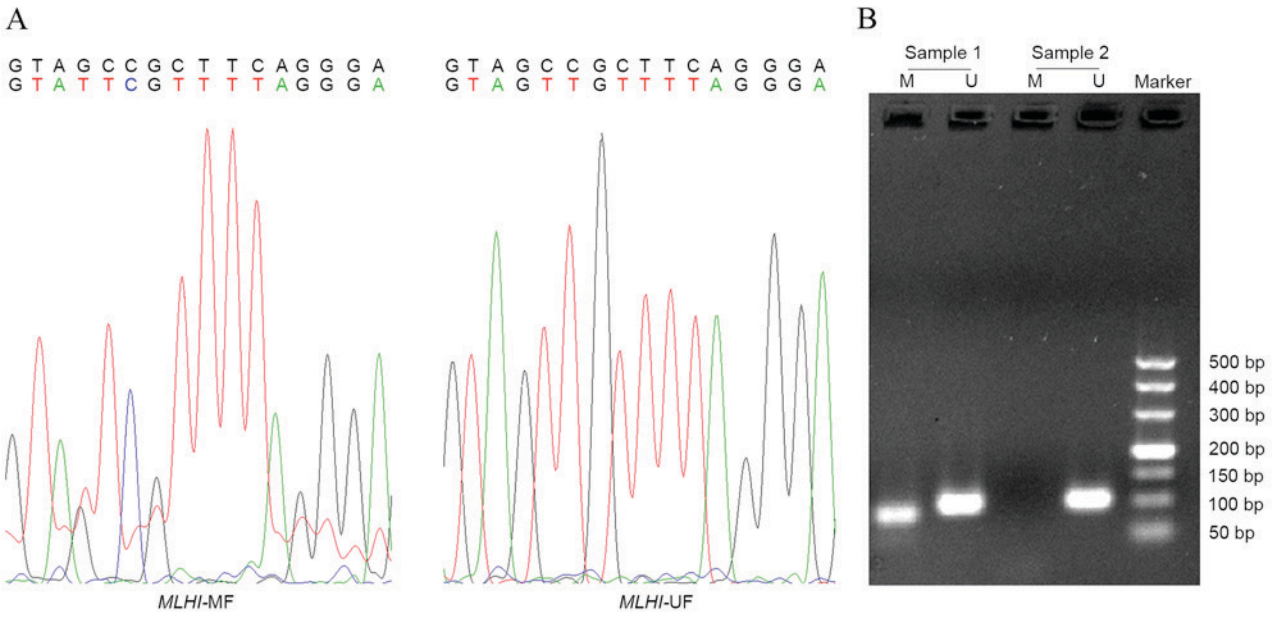

Figure 1. Representative results of sequencing validation and methylation-specific polymerase chain reaction. (A) Top row of nucleotides represents the original sequence of the gene, and the second row provides the converted sequences. (B) M and U represent methylated (91 bp) and unmethylated (102 bp) MutL homolog 1 products. *MLH1*, MutL homolog 1; *MLHI*-MF, represents the use of the forward methylated primer for sequencing; *MLHI*-UF, represents the use of the forward unmethylated primer for sequencing.

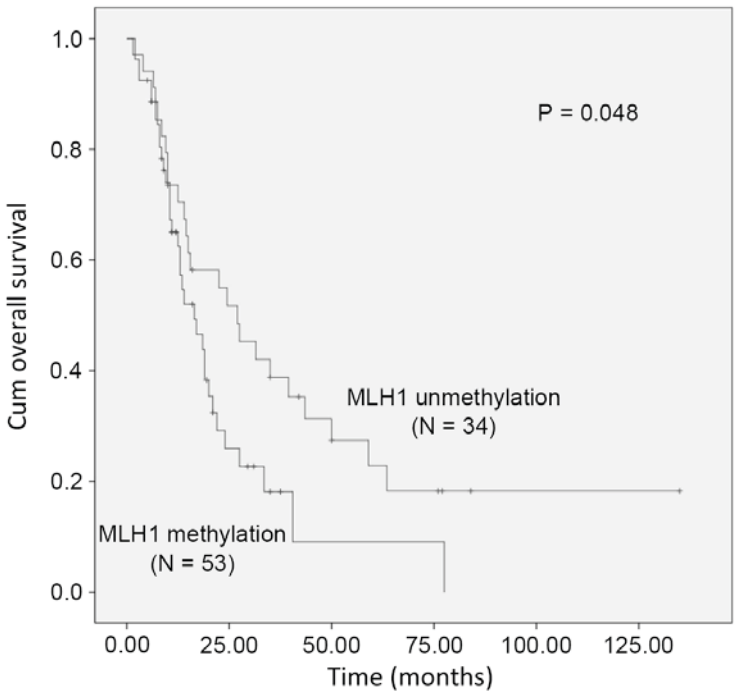

Figure 2. Kaplan-Meier survival curves comparing the survival (months) of patients with esophageal cancer and methylated or unmethylated *MLH1* promoter regions. *MLH1*, MutL homolog 1; cum, cumulative.

Discussion

In the current study, *MLH1* promoter methylation and OS was investigated in male patients with ESCC. *MLH1* promoter hypermethylation was significantly associated with poor OS in male patients with ESCC. Furthermore, diagnostic age, alcohol consumption and vascular invasion were also independent factors for the OS of patients with ESCC.

DNA mismatch repair is a conserved process that is critical for correcting any generated mismatches that escape proofreading during DNA replication (25). *MLH1* is one of the major genes involved in this process (25). The loss of the MLH1 protein may be detected in ~72% of ESCC tumors,

Table I. Baseline clinical phenotypes and *MLH1* promoter methylation in patients with ESCC.

Variables	N	*MLH1* promoter methylation	*MLH1* promoter unmethylation	P-value
Age at surgery, years				0.810
≤60	37	22	15	
>60	50	31	19	
Tumor location				0.600[a]
Upper	4	2	2	
Middle	39	22	17	
Lower	44	29	15	
Differentiation				0.439[a]
Well differentiated	8	4	4	
Moderately differentiated	46	26	20	
Poorly differentiated	33	23	10	
Lymph node metastasis				0.737
Negative	39	23	16	
Positive	48	30	18	
Disease stage				0.441
I and II	34	19	15	
III and IV	53	34	19	
Smoking history				0.486
Positive	42	24	18	
Negative	45	29	16	
Drinking history				0.335
Positive	43	24	19	
Negative	44	29	15	
Surgical margin				0.616
Negative	72	43	29	
Positive	15	10	5	
Vascular invasion				0.536
Negative	67	42	25	
Positive	20	11	9	

[a]Fisher's exact test, for all other P-values a Chi-square test was performed. *MLH1*, MutL homolog 1; N, number of patients; ESCC, esophageal squamous cell carcinoma.

concordant with *MLH1* promoter hypermethylation, which is correlated with reduced gene expression levels (31,32). *MLH1* promoter hypermethylation has been reported in numerous types of tumors, including colorectal (33), gastric cancer (34) and EC (35). Previous studies have also reported that *MLH1* hypermethylation may be associated with poor prognosis for patients with gastric (22), ovarian (36) and colorectal cancer (37), whilst studies on the prognostic value of *MLH1* methylation in Han Chinese patients with EC or ESCC are scarce. The present study demonstrated that *MLH1* promoter hypermethylation is a prognostic marker of ESCC in Chinese males.

The epidemiology of ESCC demonstrates that there is a high gender bias, evidenced by a gender ratio of 8-9:1 in favor of males (38). In addition, the consumption of alcohol and cigarettes occurs more frequently in males, as compared with in females in China (39,40). Although the present study did not examine whether alcohol and cigarette consumption were risk factors for ESCC, as investigated in previous studies (41,42), the results demonstrated that the consumption of alcohol was significantly associated with poor OS in ESCC. Vascular invasion has previously been significantly correlated with ESCC (43). In the present study, patients with vascular invasion had a significantly shorter survival time, compared with patients without, suggesting a role for vascular invasion in the progression and prognosis of ESCC.

In conclusion, the present study identified *MLH1* promoter methylation as a prognostic marker of ESCC. It was also demonstrated that patients with ESCC significantly benefited from early-stage diagnosis. Alcohol consumption, smoking and vascular invasion may be involved in the progression of ESCC; however, further studies are required to investigate these findings.

Table II. Survival analysis of 87 patients with esophageal cancer with MSP detection of *MLH1* promoter methylation.

Variables	Median OS, (months)	χ^2	Univariate P-value	HR (95% CI)	Multivariate P-value
Age at surgery, years			0.028[a]		0.011[a]
≤60	31.5	4.812		0.486 (0.279-0.849)	
>60	14.0				
Tumor location			0.835		
Upper	31.5	0.360			
Middle	19.0				
Lower	17.0				
Differentiation			0.138		
Well differentiated	50.0	3.965			
Moderately differentiated	21.0				
Poorly differentiated	14.0				
Lymph node metastasis			0.211		
Negative	24.5	1.566			
Positive	14.5				
Disease stage			0.213		
I and II	21.0	1.551			
III and IV	14.5				
Smoking history			0.307		
Positive	17.0	1.042			
Negative	20.0				
Drinking history			0.015[a]		0.013[a]
Positive	13.0	5.880		1.968 (1.157-3.348)	
Negative	22.0				
Surgical margin			0.421		
Negative	19.0	0.647			
Positive	14.5				
Vascular invasion			0.015[a]		0.047[a]
Positive	12.5	5.903		1.791 (1.007-3.185)	
Negative	22.5				
Methylated status of *MLH1* promoter			0.048[a]		0.047[a]
Methylation	16.5	3.902		1.716 (1.008-2.921)	
Unmethylation	27.0				

[a]P<0.05. *MHL1*, MutL homolog 1; OS, overall survival; HR, hazard rate; MSP, methylation-specific polymerase chain reaction.

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