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A promoter polymorphism in the hMLH1 gene (-93G/A) associated with sporadic colorectal cancer

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Abstract. Colorectal cancer (CRC) is a worldwide problem for public health. mutL homolog 1 (MLH1) is a key component of the mismatch repair system, and the MLH1-93G/A polymorphism (rs1800734) is predicted to affect MLH1 protein expression, suggesting that the polymorphism may be associated with the cancer risk; however, the results concerning this have been inconsistent. In order to investigate the possible correlation between human (h)MLH1-93G/A polymorphism and the development and progression of sporadic CRC (SCRC) in China, the genotypes of hMLH1-93G/A were detected by the TaqMan MGB probe method in 312 SCRC patients and 300 healthy controls, and immunohistochemical staining was also performed to measure the expression of hMLH1 in cases with different alleles among the SCRC patients and normal controls. It was observed that the A/A genotype and A allele significantly increased the risk of developing Duke's stage C+D CRC and lymphatic metastasis. hMLH1 expression of the A allele was lower than that of the G allele in CRC. By contrast, there was no statistically significant difference in hMLH1 expression for the A allele and the G allele in the normal controls. These results suggested that hMLH1-93G/A polymorphism may not be associated with the overall risk of CRC, but that the hMLH1-93A/A genotype and A allele are associated with the progression of CRC.

Introduction

Colorectal cancer (CRC) is a worldwide problem for public health, and is becoming more prevalent in Asian countries, particularly China (1). CRC is a prototypic model for the genetic basis of cancer. Alterations in the DNA mismatch repair (MMR) pathway have been causally linked to its etiology. The MMR pathway is one of the major DNA repair pathways; it plays an important role in repairing single-base mismatches and in insertion-deletion loops, which result from slippage during DNA replication (2,3). More and more MMR genes are being found to contain common single nucleotide polymorphisms (SNPs), which can predispose individuals to non-familial CRC with low to moderate penetrance (4-6). mutL homolog 1 (MLH1), which is located on chromosome 3p22.2, is a key component of the MMR system; it is involved in mismatch strand excision and subsequent repair (7), while recruiting other mismatch repair proteins to the mismatch sites to correct the errors during DNA replication (8,9). The MLH1-93G/A polymorphism (rs1800734) is located in the core promoter region, which is essential for maximum transcriptional activity. Polymorphism variants in this region are predicted to affect MLH1 protein expression (10,11). It was previously reported that the loss of MLH1 proteins expression had been associated with the susceptibility of several cancers. Based on these observations, particularly in view of the importance of MLH1 in colorectal carcinogenesis, we hypothesized that the polymorphism in the MLH1 gene may modulate the risk of CRC. Thus, the present matched case-control study was performed to investigate whether any associations exist between the -93G>A polymorphism and SCRC in China. In addition, immunohistochemical staining was used to measure the expression of MLH1 protein in cases with different alleles, which including CRC and normal control cases, in order to check the function of the -93G>A polymorphism.

Materials and methods

Approval and consent. The Institutional Review Boards of the Drum Tower Hospital (Nanjing, Jiangsu, China) approved the study and written informed consent was obtained from all participants.

Study population. The case-control study included 312 SCRC patients (age range, 19-89 years; mean age, 60.52±16.38 years; male/female, 193/119) and 300 normal healthy controls (age range, 23-78 years; mean age, 58.64±12.33 years; male/female, 169/131) recruited from Wuxi No. 2 People's Hospital (Wuxi, China) between January 2006 and October 2010. All patients were diagnosed with pathologically confirmed CRC and 284 patients received surgery. The 300 control subjects were randomly selected from the Center of Physical Examination

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(Wuxi No. 2 People's Hospital) from patients who had no history of malignancy during the same time period of the case recruitment. None of the subjects were blood-related. All the subjects were interviewed to obtain information on their sociodemographic characteristics, dietary habits, smoking and drinking status, and their individual and family history of cancer. SCRC cases and controls were matched for age, gender, and smoking and drinking history.

Genotyping analysis. The National Center for Biotechnology Information SNP database (http://www.ncbi.nlm.nih.gov/snp/) and related literature was searched to identify functional SNPs in human (h)MLH1. The criteria for SNP selection were as follows: i) A minor allele frequency of >0.05 in the Chinese population; ii) a genotype call rate of ≥95%; and iii) SNPs that have been closely associated with tumorigenesis. The MLH1-93G>A polymorphism (SNP rs1800734) was selected for genotyping. Genomic DNA was extracted from the peripheral blood samples of all subjects using a purification kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The MLH1-93G>A polymorphism was genotyped using the TaqMan assay on an Applied Biosystems® 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The PCR cycling conditions were as follows: 50 cycles at 95˚C for 10 min, 95˚C for 15 sec and 60˚C for 50 sec. The primer sequences were as follows: Forward, 5'-ACCCAGCAACCC ACAGAGT-3' and reverse, 5'-GTCTAGATGCTCAACGGA AGTG-3'.

Immunohistochemical staining for MLH1. CRC tissue samples were collected from 60 CRC patients who underwent surgery. Normal colon tissue samples were collected from 56 outpatients via colonic biopsies during colonoscopy examination. Immunohistochemistry was performed using the avidin-biotin-peroxidase complex method (12). Immunohistochemical staining was performed on the 4-μm thick paraffin-embedded tissues, which were mounted on positively-charged slides. The tissue sections were incubated with primary mouse monoclonal antibody against MLH1 (dilution, 1:50; cat. no. ab14206; Abcam, CA, USA) overnight at 4˚C, followed by the biotin-conjugated goat anti-mouse IgG H&L secondary antibody (dilution, 1:1,000; cat. no. ab6788; Abcam) for 30 min at 37˚C. Images were captured using an Olympus-BX50F4 microscope (Olympus, Tokyo, Japan) and quantitatively analyzed using the Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA). The normal colorectal tissues were used as positive controls for MLH1 staining. Negative controls were run without the primary antibody.

Statistical analysis. Standard χ^2 tests were used to determine the differences in allelic and genotypic frequencies between SCRC patients and control subjects in the case-control study. Student's t-test was used to compare MLH1 expression between the G and A alleles. Allele and genotype proportions were tested for Hardy-Weinberg equilibrium. The genotype data were further stratified by gender, age, smoking history, alcohol intake, tumor location and size, differentiation, Duke's stage (13), and lymphatic and distant metastasis status of

Table I. Distribution of alleles and genotypes for MLH1-93G/A (single nucleotide polymorphism rs1800734) in sporadic colorectal cancer patients and controls.

		Allele, n (%)			Genotype, n (%)				
Subjects	Number	G	A	P-value	GG	GA	AA	P-value	HWE-p
All cases	312	271 (43.4)	353 (56.6)	0.879	66 (21.2)	139 (44.6)	107 (34.2)	0.219	0.099
All control	300	258 (43.0)	342 (57.0)		52 (17.3)	154 (51.3)	94 (31.3)		0.418
Female cases	119	107 (45.0)	131 (55.0)	0.503	27 (22.7)	53 (44.5)	39 (32.8)	0.774	0.275
Female controls	131	110 (42.0)	152 (58.0)		25 (19.1)	60 (45.8)	46 (35.1)		0.494
Male cases	193	164 (42.5)	222 (57.5)	0.725	39 (20.2)	86 (44.6)	68 (35.2)	0.110	0.220
Male controls	169	148 (43.8)	190 (56.2)		27 (16.0)	94 (55.6)	48 (28.4)		0.090

MLH1, mutL homolog 1; HWE-p, Hardy Weinberg equilibrium P-value.

Table II. Stratified analyses between the human mutL homolog 1 (rs1800734) genotypes and the sporadic CRC risk.

Subjects	Genotype, n (%)			χ^2	P-value
	GG	GA	AA		
Controls	52 (17.3)	154 (51.3)	94 (31.3)	3.036	0.219
Patients[a]	66 (21.2)	139 (44.6)	107 (34.2)		
Gender				0.344	0.842
Male	39 (20.2)	86 (44.6)	68 (35.2)		
Female	27 (22.7)	53 (44.5)	39 (32.8)		
Age, years				4.553	0.103
>60	29 (19.6)	60 (40.5)	59 (39.9)		
≤60	37 (22.6)	79 (48.2)	48 (29.2)		
Smoking				0.759	0.684
Yes	36 (23.4)	65 (42.2)	53 (34.4)		
No	26 (20.0)	61 (46.9)	43 (33.1)		
Alcohol intake				3.971	0.137
Yes	35 (21.4)	66 (40.2)	63 (38.4)		
No	27 (22.5)	60 (50.0)	33 (27.5)		
Tumor location				6.653	0.155
Proximal[b]	18 (19.6)	46 (50.0)	28 (30.4)		
Distal[c]	16 (18.2)	34 (38.6)	38 (43.2)		
Rectal	28 (26.9)	46 (44.2)	30 (28.9)		
Tumor size, cm				1.176	0.555
<5	37 (21.3)	74 (42.5)	63 (36.2)		
≥5	25 (22.7)	52 (47.3)	33 (30.0)		
Differentiation				3.154	0.532
Well and moderately	47 (21.9)	90 (41.9)	78 (36.2)		
Poorly	8 (19.5)	22 (53.7)	11 (26.8)		
Other[d]	7 (25.0)	14 (50.0)	7 (25.0)		
Duke's stage				8.360	0.015
A/B	28 (27.2)	51 (49.5)	24 (23.3)		
C/D	34 (18.8)	75 (41.4)	72 (39.8)		
Lymphatic metastasis				6.590	0.031
Yes	32 (18.6)	72 (41.9)	68 (39.5)		
No	30 (26.8)	54 (48.2)	28 (25.0)		

Table II. Continued.

Subjects	Genotype, n (%)			χ^2	P-value
	GG	GA	AA		
Distant metastasis				4.794	0.091
Yes	10 (21.3)	27 (57.4)	10 (21.3)		
No	52 (21.9)	99 (41.8)	86 (36.3)		

[a]The study included 312 CRC cases, only 284 cases were available for the data on the clinical variables of smoking, alcohol intake, tumor location and size, differention, Duke's stage, and lymphatic and distant metastasis status. [b]Ascending colon, transverse colon. [c]Descending colon, sigmoid colon. [d]Other included mucinous adenocarcinoma, signet-ring cell carcinoma and adenosquamous carcinoma CRC, colorectal cancer.

CRC. The statistical tests were analyzed by SPSS 16.0 system software (SPSS Inc., Chicago, IL, USA) and a two-tailed significance level of $P<0.05$ was used.

Results

Table I shows the allele and genotype distributions for SCRC patients and controls. The allele and genotype proportions were in Hardy-Weinberg equilibrium (P=0.099 and P=0.418, respectively). Meanwhile, the allele and genotype frequencies between the SCRC patients and controls were compared, and no significant differences were observed in either the allele (P=0.879) or genotype (P=0.219) frequencies. When the CRC patients were stratified by gender, age, smoking history, alcohol intake, tumor location and size, differentiation, Duke's stage, and lymphatic and distant metastasis, no association was found between the rs1800734 polymorphism and the clinical variables of gender, age, smoking, alcohol intake, tumor location and size, differentiation or distant metastasis in SCRC patients (all $P>0.05$). However, stratifying the samples by Duke's stage and lymphatic metastasis, significant differences were found in the allele (χ^2 test, P=0.004) and genotype (χ^2 test, P=0.015) frequencies (Tables II and III). In addition, there were also a significant association between the allele (χ^2 test, P=0.010) and genotype (χ^2 test, P=0.031) frequencies and lymphatic metastasis (Tables II and III). The frequency of the A/A genotype and allele A was higher in Duke's stage C+D SCRC patients than in Duke's stage A+B SCRC patients. The frequency of the A/A genotype and allele A was also higher in the SCRC patients with lymphatic metastasis than in the SCRC patients without lymphatic metastasis.

Immunohistochemical staining for MLH1 was evaluated in 60 SCRC patients (G/G vs. G/A vs. A/A: 17 vs. 26 vs. 17) and 56 normal controls (G/G vs. G/A vs. A/A: 18 vs. 22 vs. 17) (Fig. 1). MLH1 expression levels with the different alleles were compared between the CRC patients and the normal controls, as well as between Duke's stage A+B and C+D SCRC patients (Fig. 2). MLH1 expression was significantly higher in the normal controls, in Duke's stage A+B patients and in G allele SCRC patients than in SCRC patients (P=0.029), Duke's stage C+D patients (P=0.001) and G allele SCRC patients (P=0.018), respectively. By contrast, there was no statistically significant difference in hMLH1 expression for the A and G alleles in the normal controls (P=0.965).

Discussion

Several studies have confirmed that hMLH1 plays an important role in CRC, while SNPs of mismatch repair genes are believed to provide important information for the diagnosis of CRC (14). However, to the best of our knowledge, few studies on the association between SNPs of MMR genes and sporadic CRC (SCRC) in China are available. Thus, in the present study, it was proposed that the SNP of the hMLH1 gene was linked to CRC.

The genotype distribution of the SNP MLH1-93G/A (rs1800734) has shown differences among varying ethnic populations. The frequency of polymorphism -93G/G was found to be higher than other polymorphisms in European

Table III. Allellic distribution of human mutL homolog 1-93G/A (single nucleotide polymorphism rs1800734) with regard to Duke's stage and lymphatic metastasis status among 284 sporadic colorectal cancer cases.

Parameter	Cases	Allele		χ^2	P-value	OR (95% CI)
		G	A			
Duke's stage					0.004	
A/B	103	107 (51.9)	99 (48.1)	8.244		1.655 (1.172-2.337)
C/D	181	143 (39.5)	219 (60.5)			
Lymphatic metastasis					0.010	
Yes	172	136 (39.5)	208 (60.5)	6.590		0.642 (0.458-0.901)
No	112	114 (50.9)	110 (49.1)			

OR, odds ratio; CI, confidence interval.

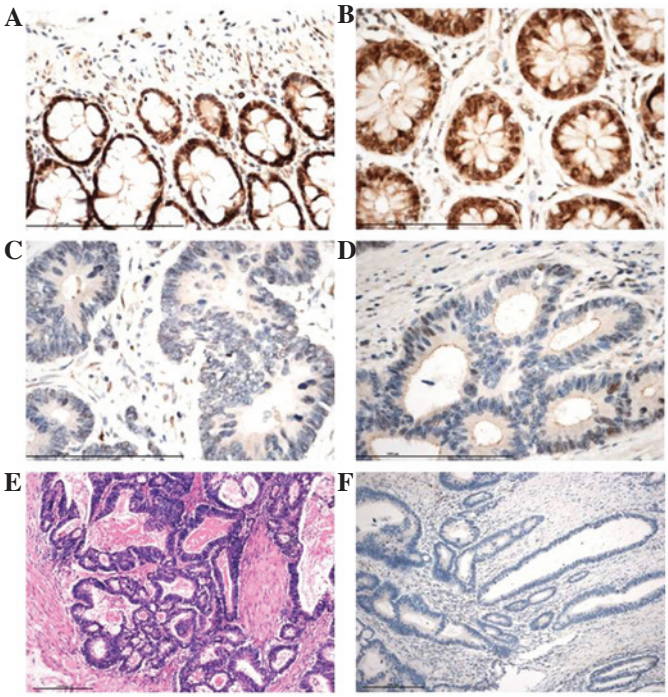

Figure 1. Immunohistochemical staining for MLH1. (A and B) MLH1 expression in normal colorectal tissue with the genotypes (A) G/G and (B) G/A (magnification, x400); (C and D) MLH1 expression in moderately-differentiated CRC with the genotypes (C) G/A and (D) A/A (magnification, x400); (E) hematoxylin and eosin staining in CRC tissue (magnification, x100); and (F) negative control (x100). MLH1, mutL homolog 1; CRC, colorectal cancer.

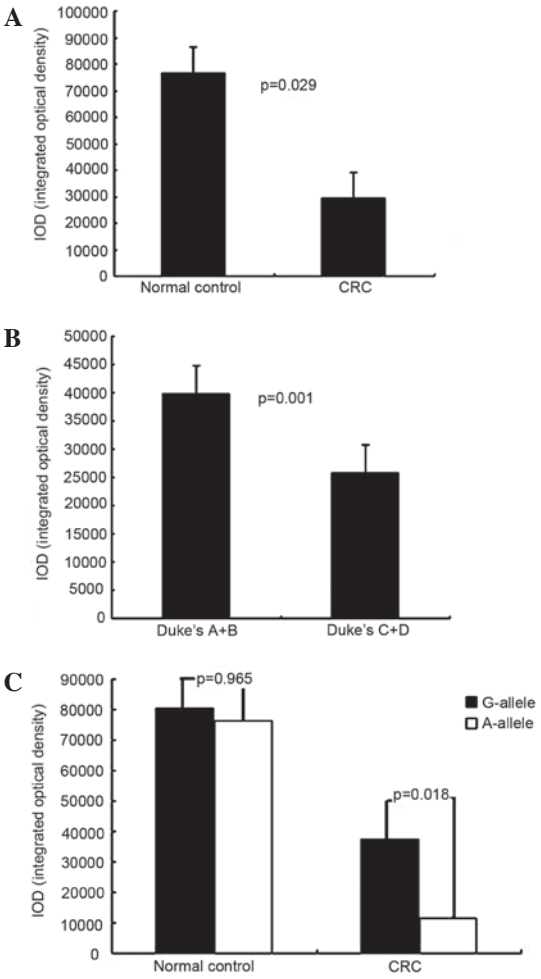

Figure 2. MLH1 expression in colorectal tissues of different types. (A) MLH1 expression is signifcantly higher in normal controls than in CRC patients; (B) MLH1 expression is signifcantly higher in Duke's A+B CRC patients than in Duke's C+D CRC patients; (C) There is no significant difference in MLH1 expression between the G and A alleles in the normal controls. However, MLH1 expression is significantly higher for the G allele than for the A allele in the CRC patients. MLH1, mutL homolog 1; CRC, colorectal cancer.

and North-American populations (15). However, in the present study, the G/A frequency in normal controls and CRC cases was observed to be higher than others. This was in agreement with other studies on Asian populations (16,17). These discrepancies may be explained by genetic variation in the different ethnic groups of the various study populations.

It is notable that the MLH1-93G/A variant has been associated with several cancers. For example, the MLH1-93A allele has been positively associated with the risk of developing MMR-deficient CRC, particularly CRC with somatic loss of MLH1 protein expression (18), and the risk of microsatellite instability (MSI)-positive colon cancer (19). A previous study also suggested that the -93A allele was associated with an increased risk of MSI-high, but not microsatellite-stable, colorectal tumors (19). It was also reported that the -93A allele

of the MLH1 gene was associated with the risk for squamous cell lung cancers in Korean patients with a gene-smoking interaction (16). In the present study, it was found that the MLH1-93G/A variant was not associated with the risk of SCRC overall, which is consistent with the results of a study by Campbell *et al* (15). However, the frequency of the A allele was significantly higher in Duke's stage C+D SCRC patients and SCRC patients with lymphatic metastasis, compared with Duke's stage A+B SCRC patients and those without lymphatic metastasis, respectively. Furthermore, MLH1 expression was lower in the Duke's stage C+D SCRC patients, and the MLH1 expression was lower for the A-allele than for the G-allele in the CRC patients. Considering these findings, the MLH1-93G/A polymorphism did not appear to affect MLH1 expression in the normal controls. However, it may play a role in the expression of MLH1 following SCRC formation, and it may be associated with the tumor progression of CRC.

The molecular mechanisms responsible for the involvement of MLH1 in CRC progression remain unclear. The MLH1-93G/A polymorphism is located in the MLH1 CpG island, at -93 nucleotides from the transcription start site in the core promoter region (10). There are two transcription binding sites, nuclear factor for interleukin-6 expression and GT-IIB trihelix transcription factor, harbored in this region, which are required for maximal transcriptional activity (10). Based on this knowledge, it is possible that the -93 A allele is susceptible to MLH1 abnormal methylation and gene silencing as a result of altered transcription factor binding. As aforementioned, polymorphism in this region is predicted to regulate MLH1 protein expression. We suggest that the -93G to A transition could plausibly reduce MLH1 gene transcription and expression by altering its epigenetic status, thereby reducing the DNA repair capability. A study by Chen *et al* showed an association between the MLH1 -93A allele and the methylation of the MLH1 promoter in CRC and endometrial cancer (20). Recent studies have suggested that site-specific repressors of transcription may recruit DNA methyltransferases (21,22).

In conclusion, in the present study, an association was found between the MLH1-93A allele variant and the elevated risk of Duke's stage C+D CRC. Furthermore, the A-allele may be a repressive factor for the transcriptional activity of MLH1, and thereby affect MLH1 expression. However, the manner via which the variation affects the risk for epigenetic silencing and has a possible affect on the progression of CRC remains undetermined. In view of this, further studies and larger sample sizes are required to confirm these findings.

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