Molecular analysis of *MLH1* variants in Chinese sporadic colorectal cancer patients

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ABSTRACT. Single nucleotide polymorphisms (SNPs) in mismatch repair genes, especially in the *MLH1* gene, are closely associated with susceptibility to hereditary nonpolyposis colorectal cancer. However, few relevant findings are available regarding the association between sporadic colorectal cancer (SCRC) and SNPs of *MLH1* in Chinese patients. Therefore, the present study aimed to describe the pathogenic association between three important *MLH1* polymorphisms and SCRC in the Chinese population. Peripheral blood samples from 156 SCRC patients and 311 healthy controls were collected. DNA was

purified from peripheral blood, and the V384D, R217C, and I219V polymorphisms were evaluated using high-resolution melting analysis and direct sequencing. The association between the three important *MLH1* polymorphisms and clinical pathological features of the SCRC patients was analyzed. In addition, PMS2-MLH1 protein interactions were determined by co-immunoprecipitation (Co-IP) to determine the protein functional alteration induced by these SNPs. Among the three polymorphisms, V384D was significantly associated with the risk of SCRC (OR = 31.36, P < 0.0001). The allele frequencies were 4.81 and 0.16% in the SCRC group. No association was found between SCRC and R217C, or between SCRC and I219V. Moreover, the allele frequency of R217C was significantly higher in the SCRC patients younger than 60 years than in those older than 60 years. Co-IP showed that the *MLH1* R217C, V384D, and I219V variants had relative binding abilities with PMS2 of 0.59, 0.70, and 0.80, respectively, compared with the wild-type. These findings suggest that *MLH1* V384D could be a promising genetic marker for susceptibility to SCRC.

Key words: Single nucleotide polymorphism; Colorectal cancer; High-resolution melting; Mismatch repair gene

INTRODUCTION

Colorectal cancer (CRC) is the fourth most frequently diagnosed cancer worldwide. It has been estimated that the number of deaths due to CRC will reach approximately 376,700 by 2020 in Asia (Ferlay et al., 2010). CRC is traditionally divided into sporadic colorectal cancer (SCRC) and familial (hereditary) forms (Hemminki and Czene, 2002). Genetic syndromes including familial adenomatous polyposis, Peutz-Jeghers syndrome, juvenile polyposis, and hereditary nonpolyposis colon cancer (HNPCC) account for only 3% of all cases (de la Chapelle, 2004). The majority of cases are sporadic or show a pattern of familial aggregation that does not closely fit Mendelian inheritance models (Aaltonen et al., 1998). Several genetic and environmental factors contribute to the development of cancer, and it is estimated that up to 35% of all colorectal cancers are caused by a genetic predisposition (Davidson, 2007; Uccello et al., 2012; Di Rosa et al., 2013).

DNA mismatch repair (MMR) is one of the major DNA repair pathways, and is responsible for the repair of single-base mismatches and insertion-deletion loops that result from slippage during replication of repetitive sequences or during recombination (Kunkel and Erie, 2005). In the human MMR system, MLH1 and PMS2 form the heterodimeric complex MutLa, which acts as an essential matchmaker between the mismatch recognition complex and the downstream MMR factors (Jiricny and Marra, 2003). The inhibition of MLH1-PMS2 interaction or the disruption of this heterocomplex formation by MLH1 variants, including those residing outside the PMS2-binding domain, can lead to defective MMR (Nyström-Lahti et al., 2002). Germline mutations in *MLH1* were found in families with HNPCC and around 10% of SCRC patients without any familial history of the disease (Liu et al., 1996). Although the relationship between the *MLH1* gene and SCRC development has been studied in other countries around the world, we know of few published studies that address the association

between *MLH1* polymorphisms and SCRC in the Chinese population.

In recent reports, *MLH1* V384D (c.1151T>A), R217C (c.649C>T), and I219V (c.655A>A/G), located in exons 12, 8, and 8 of the *MLH1* gene, respectively, have been described as important polymorphisms in *MLH1*. In this study, to clarify the association between *MLH1* polymorphisms and SCRC in the Chinese population, we used high-resolution melting (HRM) to detect the three mutant forms of *MLH1* (V384D, R217C, and I219V) in a series of 156 cases of SCRC and 311 healthy controls. The association between the clinical pathological features of the patients and the *MLH1* variants was investigated. Furthermore, we carried out an analysis of protein-protein interactions of the MutLa complexes by co-immunoprecipitation (Co-IP) assay to assess the functional relationship between the three *MLH1* polymorphisms and SCRC in the Chinese population.

MATERIAL AND METHODS

Clinical samples

We conducted a case-control study of 467 Chinese individuals, including 156 patients with SCRC who underwent either elective or urgent surgery at the Digestive Endoscopy Center, Shanghai Tongren Hospital (Shanghai, China) between January 1, 2012 and December 31, 2014, and 311 healthy controls with no history of malignant disease. The controls were randomly selected from the same geographic region as the cases and were matched to them by age and gender. Informed consent was obtained from both patients and controls, and the Ethics Committee of the Hospital approved the study.

None of the recruited patients received preoperative chemo- or radiotherapy. Patients were also excluded if they presented a positive family history of cancer, including familial adenomatous polyposis, HNPCC, any of the HNPCC-associated cancers, multiple-colonic adenomatous polyps, or a clinical history of inflammatory bowel disease. Patients previously diagnosed with colorectal tumors or with tumors located elsewhere were also excluded.

Peripheral blood samples were collected from all patients and controls and stored in ethylenediaminetetraacetic acid (EDTA)-anticoagulant tubes. Blood obtained from the patients was stored at -80°C within 2 h of removal. The tumors were analyzed by the Pathology Department of the hospital to determine the level of differentiation and Dukes' stage. Blood samples from the healthy controls were also processed for DNA extraction immediately after their collection.

Nucleic acid purification

Genomic DNA was isolated and purified from the peripheral blood of cases and controls using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany).

Analysis of *MLH1* V384D, R217C, and I219V variants

The variant analysis was performed by polymerase chain reaction (PCR) and HRM using Rotor-Gene Q (Qiagen). The PCR-HRM product size of the *MLH1* V384D variant was 244 bp. The primer sequences were as follows: forward 5'-AATACAGACTTTGCTACCAGGACT-3' and reverse 5'-CCTAGCCCTGCCACTAGAAA-3'.

The *MLH1* R217C and I219V variants were amplified with the same pair of primers, and the PCR-HRM product sizes were 76 bp. The primer sequences were as follows: forward 5'-GGACACTACCCAATGCCTCA-3' and reverse 5'-TTATCGACATACCGACTAACAGCA-3'. All amplifications were performed in a 10-μL volume with 5 μL premix Hot Start Taq (TaKaRa, Japan), 0.3 μL SYTO-9 (X100, Life Technologies, USA), 400 nM forward primer, 400 nM reverse primer, and 10-20 ng DNA. The reaction conditions were as follows: one cycle at 95°C for 30 s; 50 cycles at 95°C for 20 s, 56°C for 20 s, and 72°C for 20 s for amplification; followed by a melt from 77° to 87° at 0.2°C per second for HRM analysis. The fluorescence difference between all other curves and the comparison curve was then plotted against temperature.

Sequencing

The samples with candidate mutations identified by HRM were confirmed by sequencing followed by an additional PCR. The PCR-sequencing primers for *MLH1* V384D were identical to those for PCR-HRM. The primers for the *MLH1* R217C and I219V variants were as follows: forward 5'-TGGGGGATGGTTTTGTTTTA-3' and reverse 5'-TCACGCCACAGAATCTAGGA-3', which produced a 441-bp amplicon. The amplification conditions were the same as in the PCR-HRM. The DNA sequencing analysis was performed in an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Plasmid constructions and cell transfection

The wild-type pcDNA3-MLH1 and pSG5-PMS2 plasmids were a gift from Dr. Guido Plotz. *MLH1* mutants V384D, R217C, and I219V pcDNA3-MLH1 were constructed using a QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer instructions. It has been reported that the human embryonic kidney fibroblast cell HEK293T is silent for hMLH1 expression because of promoter hypermethylation, and lacks hPMS2, which is not stable without hMLH1. The absence of hPMS2 allows the expression and analysis of exogenous MutLa variants in this cell line without the interference of endogenous MutLa. The 293T cells (Cells Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Science, Shanghai, China) were transiently co-transfected with pcDNA3-MLH1 and pSG5-PMS2 using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer instructions.

Co-immunoprecipitation

Total cell protein extracts were incubated with anti-MLH1 antibody (rabbit monoclonal; Abcam, Cambridge, UK) followed by immunoprecipitation with protein A+G-agarose (Beyotime Institute of Biotechnology, Haimen, China). The protein-agarose complexes were then washed, collected, and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblots were carried out according to the standard operating procedure. The densities of the desired protein bands were quantified with version 4.5.0 of the Quantity One program (Bio-Rad Laboratories, Richmond, CA, USA) and the relative amount of PMS2 was determined.

Statistical analyses

Chi-square distribution was used to analyze categorical data and $P < 0.05$ was considered statistically significant. Odds ratios (ORs) and 95% confidence intervals were calculated using the frequency of three alleles to determine risk of SCRC.

RESULTS

Genotype distributions of the three *MLH1* polymorphisms

HRM analysis revealed differences in the melting curve shape that correlated to genotype and the presence of SNP heterozygotes. Melting curves of homozygous and heterozygous products were overlaid at high temperature to visually aid comparison. Heterozygotes were easily distinguished from homozygotes by the shape of the melting curves. In addition, homozygote discrimination was based on differences in the melting temperature. Subsequently, these PCR-HRM assay results were confirmed by DNA sequencing analysis (Figure 1).

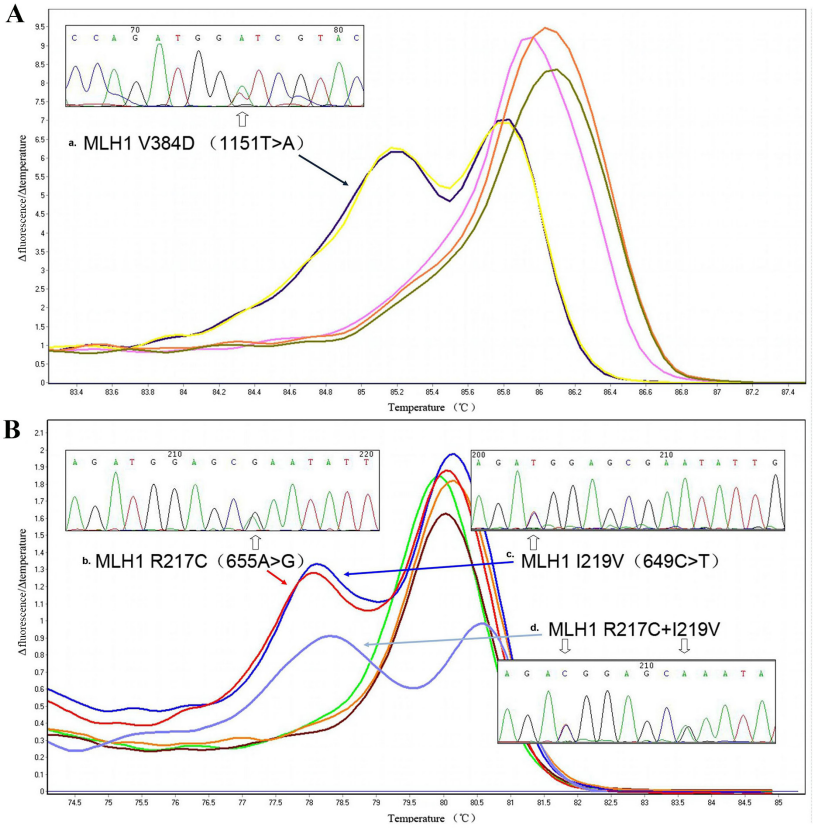

Figure 1. Results of high-resolution melting (HRM) analysis and sequencing of *MLH1* V384D, R217C, and I219V variants. **A.** Identifying *MLH1* V384D variant with HRM and sequencing in peripheral blood DNA. **B.** Identifying *MLH1* R217C and I219V variants with HRM and sequencing in peripheral blood DNA.

The genotype and allele frequencies of the three SNPs in SCRC patients and healthy controls are shown in Table 1. Genotype frequencies were in Hardy-Weinberg equilibrium in the patients and controls for all the polymorphisms analyzed. For *MLH1* V384D, the T allele was found in 5% of chromosomes from the CRC patients compared with 1% of the chromosomes from the controls (OR = 31.36, P < 0.01) (Table 1). With regards to *MLH1* R217C, the T allele was found in 2% of chromosomes from the SCRC patients compared with 1% of chromosomes from the controls (OR = 2.52, P = 0.29). For *MLH1* I219V, no statistically significant difference was found in allele frequencies between patients and controls (P = 0.29).

Table 1. Distributions of the three single nucleotide polymorphism genotypes in sporadic colorectal cancer (SCRC) patients and controls.

Group	Genotype allele	Patients (N = 156) (%)	Controls (N = 311) (%)	χ^2	P value	OR (95%CI)
V384D	AA	142 (0.91)	310 (0.99)	22.31	<E-04	0.03 (0-0.24)
	AT	13 (0.08)	1 (0.01)	20.26	<E-04	28.18 (3.81-∞)
	TT	1 (0.01)	0 (0)	0.12	0.7247	∞ (0-∞)
	A	297 (0.95)	621 (0.99)	23.96	<E-04	0.03 (0-0.23)
	T	15 (0.05)	1 (0.01)	23.96	<E-04	31.36 (4.34-∞)
R217C	CC	151 (0.97)	307 (0.99)	1.14	0.2865	0.39 (0.09-1.71)
	CT	5 (0.03)	4 (0.01)	1.14	0.2865	2.54 (0.58-11.44)
	TT	0 (0)	0 (0)	-	-	-
	C	307 (0.98)	618 (0.99)	1.13	0.2888	0.40 (0.09-1.71)
	T	5 (0.02)	4 (0.01)	1.13	0.2888	2.52 (0.58-11.21)
I219V	AA	151 (0.97)	307 (0.99)	1.14	0.2865	0.39 (0.09-1.71)
	AG	5 (0.03)	4 (0.01)	1.14	0.2865	2.54 (0.58-11.44)
	GG	0 (0)	0 (0)	-	-	-
	A	307 (0.98)	618 (0.99)	1.13	0.2888	0.40 (0.09-1.71)
	G	5 (0.02)	4 (0.01)	1.13	0.2888	2.52 (0.58-11.21)

Association between *MLH1* V384D, R217C, and I219V variants and patient characteristics

In SCRC, the frequency of R217C was higher in patients <60 years of age than in patients >60 years of age (P = 0.006). There was no statistically significant correlation between the three SNPs and gender, Dukes' stage, or differentiation in CRC samples (Table 2).

Table 2. Allele frequencies of *MLH1* V384D, R217C, and I219V variants in relation to age, gender, Dukes' stage, and differentiation in patients with colorectal cancer.

	V384D (1151A/T)			R217C (649C/T)			I219V (655A/G)		
	T	A	P value	T	C	P value	G	A	P value
Gender									
Male	9 (0.059)	143 (0.941)	0.3703	2 (0.013)	150 (0.987)	0.6942	2 (0.013)	150 (0.987)	0.6942
Female	6 (0.038)	154 (0.962)		3 (0.019)	157 (0.981)		3 (0.019)	157 (0.981)	
Age (years)									
<60	4 (0.039)	98 (0.961)	0.6101	5 (0.049)	97 (0.951)	0.0059	2 (0.020)	100 (0.980)	0.7255
≥60	11 (0.052)	199 (0.948)		0 (0.000)	210 (1.000)		3 (0.014)	207 (0.986)	
Dukes' stage									
A	4 (0.039)	98 (0.961)	0.613	5 (0.049)	97 (0.951)	0.217	2 (0.020)	100 (0.980)	0.063
B	11 (0.052)	199 (0.948)		0 (0.000)	210 (1.000)		3 (0.014)	207 (0.986)	
C									
D	3 (0.037)	77 (0.963)		1 (0.012)	79 (0.988)		2 (0.025)	78 (0.975)	
Differentiation	4 (0.042)	92 (0.958)	0.1755	0 (0.000)	96 (1.000)	0.4557	0 (0.000)	96 (1.000)	0.8704
good/moderate	5 (0.081)	57 (0.919)		1 (0.016)	61 (0.984)		3 (0.048)	59 (0.952)	
poor	3 (0.041)	71 (0.959)		3 (0.041)	71 (0.959)		0 (0.000)	74 (1.000)	

Co-IP analysis for the interaction between *MLH1* variants and PMS2

To assess directly the impact of the polymorphisms of *MLH1* on MLH1-PMS2 heterodimerization, we measured the amount of PMS2 that was co-precipitated when MLH1 was immunoprecipitated from the cell extracts (Figure 2). However, the three *MLH1* variants displayed varying degrees of PMS2 binding inhibition. Co-IP showed that the relative binding abilities of *MLH1* R217C, V384D, and I219V towards PMS2 were 0.59, 0.70, and 0.80, respectively, compared with the wild-type.

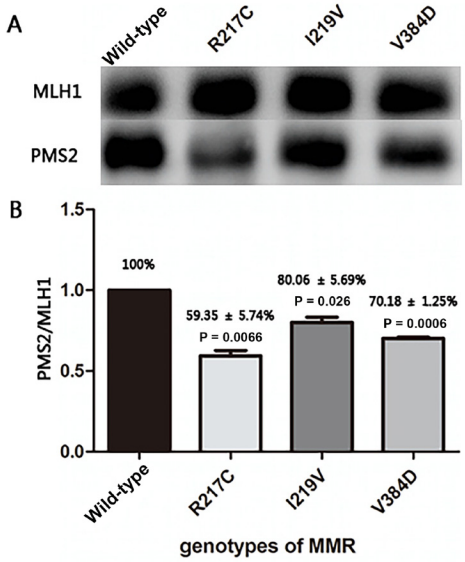

Figure 2. Co-immunoprecipitation (Co-IP) results for MLH1 V384D, R217C, and I219V variants. Co-IP of MLH1 and PMS2 showed lower quantities of PMS2 binding with MLH1 in the R217C, V384D, and I219V variants than in the wild-type, which suggests dysfunctions in the three variants.

DISCUSSION

It is widely accepted that the mutations of MMR genes, especially *MLH1*, are risk factors for the development of HNPCC (Houlston and Tomlinson, 2001). Although the relationship between the *MLH1* mutation and SCRC has been studied in other countries around the world, few published studies have focused on this correlation with SCRC in the Chinese population. In this study, we established that the *MLH1* V384D polymorphism is associated with an increased risk of developing SCRC in the Chinese population, probably in part because it impairs the interaction between MLH1 and PMS2.

The *MLH1* V384D (1151A/T) polymorphism has frequently been detected in East Asian HNPCC patients (Han et al., 1996; Lee et al., 2005b; Yap et al., 2009). In our study, the presence of the *MLH1* V384D variant was significantly higher in CRCs (14/156) than in healthy controls (1/311). Thus, the frequency of the V384D mutant was 27-fold higher in the SCRC group than in the control group ($P < 0.01$). Ohsawa et al. (2009) reported that the V384D variant was detected in 40 (6.0%) of the 670 CRC patients and 5 (1.5%) of the

332 controls in a Japanese population. Our results highlighted the differences in genetic background with regards to *MLH1* in colorectal cancer susceptibility in these two populations. We found no difference in the pathological characteristics between the V384D carriers and the non-V384D carriers in the SCRC group. Because the MMR activity of the V384D variant is influenced by its interaction with PMS2, a co-immunoprecipitation assay was used to detect the PMS2 binding effects of the MLH1 variants. Compared with wild-type MLH1, MLH1 V384D showed an obviously reduced interaction with PMS2 (70.18%) by Co-IP assay. The impaired protein-protein interaction of these MMR protein complex suggested an increased risk of developing SCRC for the *MLH1* V384D mutation carriers.

A recent study found that the *MLH1* V384D allele was over-represented in patients with epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) resistance. Patients with the *MLH1* V384D mutation had a significantly shorter progression-free survival. *MLH1* V384D was identified as a novel molecular marker for a genetic predictor of primary EGFR-TKI resistance in EGFR L858R-positive lung adenocarcinomas (Chiu et al., 2015). This finding indicates that MLH1 may have MMR-independent functions involved in EGFR signaling or other proliferation or survival pathways. Further investigation is needed to elucidate the impact of *MLH1* V384D on drug resistance in colorectal cancer.

The *MLH1* R217C (649C>T) polymorphism was first detected in a Japanese HNPCC patient (Miyaki et al., 1995). In our study, although the differences did not achieve significance ($P = 0.28$), a higher genotypic or allelic frequency of *MLH1* R217C was detected in the SCRC patients than in the controls (5/156 and 4/311, respectively). Furthermore, we found that CRC patients younger than 60 had a higher *MLH1* R217C incidence than patients older than 60, suggesting an association between *MLH1* R217C and a high risk of early-onset colorectal cancer. In addition, our data suggested that this variant may be pathogenic owing to the functional impairment of the MLH1 protein in its interaction with PMS2, as shown by Co-IP analysis. Ellison et al. (2004) remarked that *MLH1* R217C is an efficiency polymorphism because it functions in DNA MMR at a reduced efficiency. Further studies with larger sample sizes would be necessary to confirm the relationship between the R217C polymorphism and the age of disease onset in Chinese SCRC patients.

The *MLH1* I219V (655A/G) polymorphism results in an amino acid change from Ile to Val. Case-control studies have shown that this Ile/Val219 site influences the risk of developing a variety of cancers including ovarian (Mann et al., 2008), lung (An et al., 2008), and breast cancer (Lee et al., 2005a). Milanizadeh et al. (2013) found that the *MLH1* I219V polymorphism is associated with SCRC susceptibility ($P = 0.01$). Contrary to their result, our study showed that there was no difference in the frequency of *MLH1* I219V between the SCRC patients and the controls. This divergence may be attributed to the substantial ethnicity-related diversity in the genetic polymorphism. Co-IP showed a moderate reduction of binding ability (80%) between MLH1 I219V and PMS2 compared with the wild-type MLH1 and the R217C variant.

In conclusion, the *MLH1* V384D SNP may contribute to an increased risk of SCRC in the Chinese population, and an ethnic difference is evident in terms of *MLH1* mutation frequency between East Asian and Western populations.

Conflicts of interest

The authors declare no conflict of interest.

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