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Original article

The relationship between human muth homolog 1 gene mutation at site 415 and sporadic colon cancers in Chinese Han population

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Background: The genetic factors of colon cancer play an important role in the tumor development and growth. The incidence of colon cancers has greatly increased in China. However, few data is available for the relationship between human muth homolog 1 (hMLH1) gene mutation at site 415 and sporadic colon cancers in Chinese population.

Objective: Investigate the relationship between G→C mutation in hMLH1 gene at site 415 and sporadic colon cancers in Chinese Han population.

Methods: Using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and DNA sequencing techniques, the genotype of the hMLH1 gene was analyzed at site 415 in 97 cases of sporadic colon cancer patients and 138 controls. Reverse-transcription (RT)-PCR was used to determine the level of hMLH1 mRNA expression in normal colonic mucosa of patients with different genotype.

Results: The frequency of genotype C/C at the 415 site of the hMLH1 gene was significantly higher in colon cancer patients than in controls. The expression levels of hMLH1 mRNA in normal colonic mucosa were similar in colon cancer patients with different genotypes.

Conclusion: G'!C mutation in hMLH1 gene at site 415 may represent a genetic factor that is associated with sporadic colon cancer in a small group of Chinese Han population.

Keywords: human mutl homolog 1 gene, mutation, sporadic colon cancers

Intestinal polyps, chronic diarrhea, constipation, and eating habits are all risk factors for colorectal cancers (CRC) [1]. It has also been established that genetic factors play an important role in the tumor development and growth. Hereditary CRC is also known as Lynch syndrome, which is characterized by increased microsatellite instability (MSI) due to mutation in the DNA mismatch repair (MMR) genes [2, 3]. Accumulation of non-repaired post-replicational mis-incorporations in cancer-related genes may result in tumor-genesis [4].

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Human mutl homolog 1 (hMLH1) gene is one major MMR gene located on human chromosome 3p21.3. Its DNA length is 2268bp. It encodes a protein product with 756 amino acids. Mutation or methylation in this gene may cause errors in DNA MMR and result in inherited instability and susceptibility to tumor formation [5, 6]. Several researchers have shown that aberrant hMLH1 is related to inherited colon cancer, gastric cancer, endometrial cancer, chronic myelogenous leukemia, and ovarian cancer [7-9].

In China, the incidence of colorectal cancer (CRC) has significantly increased. However, the relationship between single nucleotide polymorphism (SNP) of the hMLH1 gene and the risk of sporadic colon cancers has not been well studied. In a control study between 233 patients with colon cancer and

268 healthy persons, Zhang et al. [10] found that there were significant differences of the frequency distribution of the Va1384Asp SNP in the hMLH1 gene between colon cancer patients and controls, especially in the case of younger age groups.

Lipkin et al. [11] reported that a G→C mutation (substitution of D132H amino acid) at position 415 of hMLH1 gene could increase the risk of colon cancer five-fold in Israelites (Jews). However, Shin et al. [12] and Schafmayer et al. [13] did not discover similar results in another two ethnic populations. It is unclear whether there is an association between G→C mutation at site 415 and susceptibility of sporadic colon cancers in Chinese population.

In this study, we investigated the distribution of G→C mutation in hMLH1 gene at site 415 in 97 cases of colon cancer patients and 138 controls from Chinese Han population. Our results indicate that the G→C mutation of the mismatch repair gene hMLH1 at the site 415 is possibly one of the genetic factors that contribute to susceptibility to a small group of sporadic colon cancers in Chinese Han population.

Materials and methods

Ninety-seven patients (62 male and 35 female; mean age: 54.5±12.4 years) with sporadic colon cancer were recruited from Cancer Hospital of Hubei Province and Renmin Hospital of Wuhan University. They consisted of both in- and out-patients visiting Han population over a three-year period between March 2005 and March 2008. The clinical diagnoses of colon cancer were confirmed by post-operation pathological examination. All patients enrolled in the present study were free of familial history of colon cancer, metastatic carcinoma, anal cancer, malignant lymphoma, and gastrointestinal stromal tumor. This study was approved by the Ethics Committee of Renmin Hospital Wuhan University.

The baseline clinical characteristics and pathological results of these patients are summarized in **Table 1**.

Table 1. Clinical data of colon cancer patients (n=97).

Categories	Number (male/female)
Stages*	
I	14 (9/5)
II	20 (13/7)
III	26 (17/9)
IV	37 (23/14)
Pathological classification	
G1: highly differentiated	19 (14/5)
G2: moderately differentiated	54 (34/20)
G3: poorly differentiated	18 (10/8)
G4: undifferentiated	6 (4/2)
Tumor size	
28 mm	19 (10/9)
29-39 mm	25 (15/10)
40-60 mm	12 (8/4)
>60 mm	4 (3/1)
unclear	37 (26/11)
Tumor site	
Cecum	9 (7/2)
Appendix	1 (0/1)
Ascending colon	8 (5/3)
Hepatic flexure	8 (6/2)
Transverse colon	13 (8/5)
Splenic flexure	7 (6/1)
Descending colon	15 (9/6)
Sigmoid colon	17 (9/8)
Rectum	19 (12/7)

*Clinical phases I-IV are a modified classification system of Dukes A-D, which is widely used in China.

In addition, 138 normal controls (82 male and 56 female, mean age: 46.8±8.3 years) were selected randomly from Han population living in Hubei province. They underwent history and physical examinations in the People's Hospital of Wuhan University over the same period. They had no family history of colon cancer or inflammatory bowel disease. There were no significant differences between colon cancer patients and the control group in terms of age and gender (46.8±8.3 *vs*. 54.5±12.4, t=0.9, p=0.4; 82/136 *vs*. 62/97, $\chi^2 = 0.49$, p=0.5, respectively).

Genomic DNA extraction

One hundred microliter of fresh venous blood with EDTA-anticoagulant was mixed with 200 μL distilled sterile water and homogenized with a mini-grinder in 200 μL of 6M NaI (sodium iodide) for 20 seconds. Then, 400 μL of chloroform-isopropanol (24:1, v/v) was added, and the mixture was centrifuged at 12000 rpm for 10 minutes. The aqueous phase was transferred into another tube, and DNA was precipitated with isopropanol. Following centrifugation at 12000 rpm and 4°C for 10 minutes, the aqueous phase in the tube was discarded. The DNA was washed with 500 μL of 70% ethanol for twice dried at room temperature. DNA was resuspended in 40 μL of TE buffer overnight, and stored at -20°C in aliquots for future use.

PCR amplification of HMLH1 gene

The hMLH1 gene (NM_000249) was PCR amplified using the following primer set: forward, 5'-CTATTACAACGAAAACAGCTG-3; reverse, 5'-GGCTATGTTGTAAAAAAGGT-3'. All PCR reaction mixtures contained 1.5 U of Taq polymerase (TaKaRa), 10mmol/L Tris-HCl, 50 μmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 μmol/L dNTP and 80 ng primers in a final reaction volume of 50 μL. The amplification reaction was performed after an initial denaturation at 94°C for 10 minutes. This was followed by five initial cycles (94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 minutes), 30 middle cycles (94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds), and five end cycles (94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds). The final extension was 72°C for seven minutes.

Restriction endonuclease digestion of amplified fragment hMLH1 and sequencing analysis

The site 415 in hMLH1 gene corresponds to NlaIII restriction site (recognition sequence CATG at sequence 415-418). Following purification, the PCR product was subjected to NlaIII digestion for three hours. The digested product underwent electrophoresis in 3.0% agarose gel at 100V for 25 minutes. The results were observed under ultra-violet light and photographed with a gel-imaging system (Gel Doc 100 system, Bio-Rad, Hercules, USA). Some of undigested PCR products were randomly selected for DNA sequencing through an ABI 3130 sequencing system (ABI, Foster, US)

RNA extraction

One thousand microliter of pre-cooled Trizol (Invitrogen, USA) was added to fresh normal colonic mucosa tissue samples (approximately 10 mg), and was homogenized in a tissue grinder. The resultant sample was transferred into Eppendorf tube, and incubated for five minutes at 15-30°C, allowing the protein complex disintegrate completely. Added 200 μL Chloroform to the tube and gently mixed for 30 seconds. Tube was centrifuged for 15 minutes at 12,000 x g at 4°C. The supernatant (approximately 600 μL) was transferred into another tube with addition of 500 μL isopropanol. The tube was placed at room temperature for 15 minutes, followed by centrifuging for 10 minutes at 12,000 x g at 4°C. The supernatant was discarded and RNA sample in the tube bottom was washed with 1000 μL 75% ethanol twice, and dried at room temperature. The final RNA was dissolved in TE buffer and stored in -80°C freezer. The final total RNA sample was quantitated using Lambda10 Spectrophotometer (PE Co, Boston , USA), in which A260=1 was set to be 40 μL/mL of RNA and RNA concentration was calculated using the following formula:

$$\text{RNA total} = A260 \times 40 \times N.$$

RT-PCR for detection of hMHL1 gene expression

First strand of cDNA was synthesized from 50 μL of total RNA using the Access quick TMRT-PCR system (Promega Co. Madison, USA). Briefly, 25 μL 2xAccessquickTMMaster suspension liquid (Tf/DNA polymerase, dNTPs, $MgCL_2$ and reaction buffer), 3 μL 10 μM primers, 2-5 μL RNA template, and finally DEPC-treated deionized water were mixed in a sterile and RNAse-free PCR tube with a final volume of 50 μL. Then, 1 μL (5U) reverse

transcriptase was added to each reaction tube. Reverse transcription was conducted under 48°C for 45 minutes and cDNA was used as template for further PCR amplification. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included in all following PCR reactions as control (GAPDH primers: forward, 5'-CACAGTCCATGCCATCAC TG-3', reverse, 5'-TACTCCTTGGAGGCCATGTG-3'. GAPDH was also used for the normalization in the comparisons of relative amount of hMLH1 mRNA expression in samples.

Statistical methods

Continuous variables were expressed as mean± standard deviation (SD). Categorical variables were expressed as the percentage of the sample. Student t-test or ANOVA was used for the comparison between patient and control groups. Logistic regression analysis was used to calculate odd ration (OR) and 95%.confidence interval (CI). Standardization of population rate in each group was based on the data of Chinese Census in 2000, in which 51.6% were males and 48.4% were females. The Chi-square test and the Fisher exact probability test were used for the Hardy-Weinberg balance of genotype distribution. All analyses were performed using SPSS13.0 statistical software (SPSS, Chicago, USA). The differences were considered significant if p is less than 0.05.

Results

Definition of genotype of the hMLH1 gene at site 415

With the specific primers, PCR amplification generated a 140bp product for hMLH1 gene. Following enzyme NlaIII digestion, some PCR products gave rise to two smaller fragments with a size of 82 and 58bp, respectively. According to the specificity of the enzyme targeting sequence, the length polymorphism was defined as shown in **Fig. 1.** Lane 2, 3 and 4 are three bands (82bp+58bp and 140bp, partial digestion) = genotype CG, two bands (82bp+58bp, complete digestion) = genotype CC, and one band (140bp, no digestion) = genotype GG.

For confirmatory purpose, PCR products before enzyme digestion were randomly selected for DNA sequencing. Representative sequencing results for hMLH1 gene containing site 415 are shown in **Fig. 2**.

Genotype frequency of hMLH1 gene at site 415 in colon cancer patients and controls

Based on the above definition of the genotype for hMLH1 gene at site 415, the genotype frequencies were calculated and summarized in **Table 2**.

The genotype frequencies of hMLH1 gene at site 415 in patient and normal control groups were all in accordance with the Hardy-Weinberg equilibrium (data not shown, $p > 0.05$). Following standardization of population rate of sex, the phenotype frequency of CC was 7.4% in patients and 1.52% in control, which was statistically different ($p < 0.05$). To estimate the relationship between the CC phenotype and colon cancer, OR and 95%CI were calculated. The results suggest that genotype CC was significantly associated with colon cancer (OR=5.29; and 95%CI =1.07-26.04).

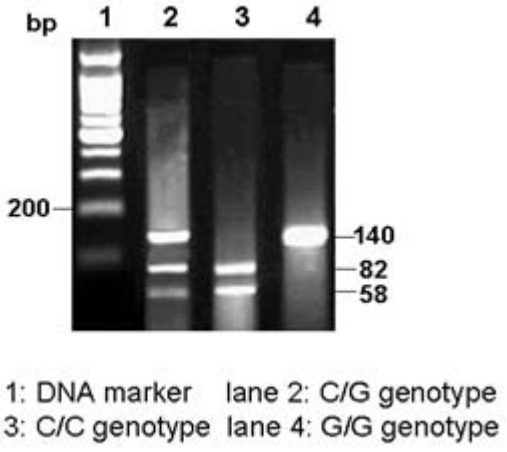

Fig. 1 Genotyping hMLH1 gene polymorphism at site 415. PCR product of hMLH1 gene at site 415 before NlaIII digestion was 140 bp. Following digestion, three genotypes were observed: CG (three bands: 140bp, 58bp and 82bp, lane 2), CC (two smaller bands: 58bp and 82bp, lane 3), and GG (one single undigested band, 140bp lane 4) (Lane 1: DNA size makers).

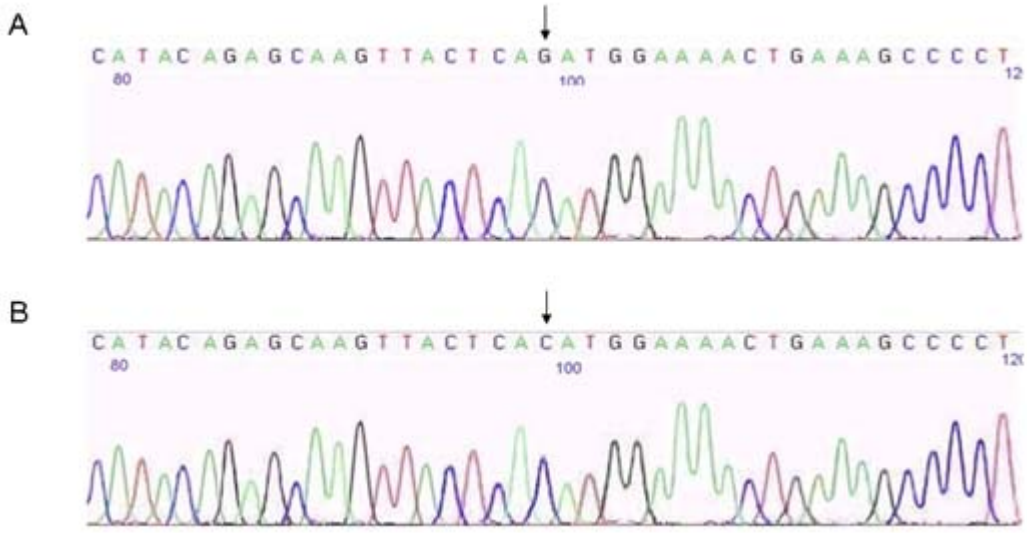

Fig. 2 Sequencing results of hMLH1 site 415 genotype. Ten cases of subjects with different genotypes from both patient and control groups were randomly selected for sequencing. The hMLH1 site 415 is indicated with arrow. Representative sequences with G and C are shown in **A** and **B**, respectively.

Table 2. Genotype frequencies of hMLH1 at site 415 in CRC patients and controls.

Genotype		Patients (n=97) Number (%)	Controls (n=138) Number (%)
	G/G	78 (80.4)	125 (90.6)
	C/G	12 (12.4)	11 (8.0)
hMLH1	C/C	7 (7.2)	2 (1.5)
	C/C*	7.4	1.5

*Sex-adjusted result, which was based on standardized population ($p < 0.05$. *vs.* control).

hMLH1 mRNA expression in patients with different genotypes of hMLH1 gene at site 415

To determine whether G→C mutation in hMLH1 gene at site 415 results in the change of hMLH1 gene expression, we examined hMLH1 gene mRNA level in colonic mucosa tissues from patients with different genotypes of site 415. Gel results indicated that hMLH1 mRNA expression levels were visually comparable in subjects with different genotypes, and the results showed a similar value (**Fig. 3**).

Discussion

As an important correcting mechanism of replication error, MMR can preserve the inherited accuracy through repairing base-base mismatch, insertion-deletion loop and heterologous mismatch during DNA replication and recombination. hMLH1 and MSH2 are the major DNA MMR genes, which are requisite in cell proliferation. When hMLH1 gene or MSH2 gene is inactive, cells will not be able to recognize and correct gene mutation and DNA replication errors. This may increase the rate and accumulation of gene mutations, which ultimately lead to the activation of onco-genesis or down-regulation of tumor suppressor genes, resulting in resistance to cell apoptosis and hence tumor formation [4, 7]. In sporadic colon cancers, the hMLH1 gene deletion rate is higher than MSH2. This suggests that hMLH1 gene mutation may play a key role in the tumorigenesis in certain sporadic colon cancer patients [14].

It has been established that SNP may influence gene expression level and/or protein structure/function. Searching and identifying gene polymorphism underlying predisposition to colon cancer is currently an intensively studied subject. Kim et al. [15] examined three SPN loci (hMLH1*1151, hMLH1*655 and

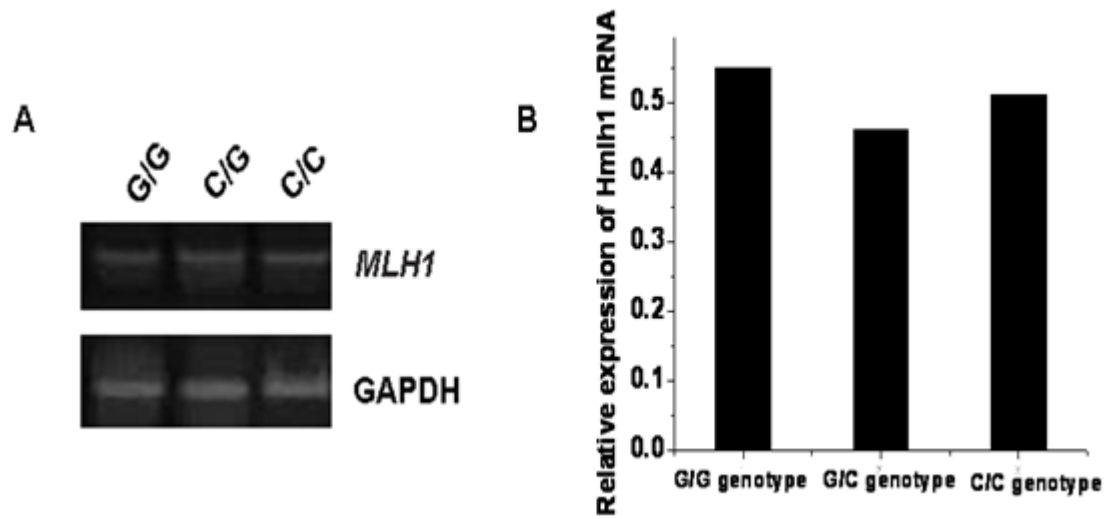

Fig. 3 hMLH1 gene expression in patients with different genotype of hMLH1 gene at site 415. **A:** Representative gel results of RT-PCR for RNA expression. **B:** Quantitated results of scanned gel results.

hMSH2*1168) in MMR genes. None of them showed association with sporadic colon cancer. Lipkin et al. [10] reported that G→C mutation (substitution of D132H amino acid) in hMLH1 gene at site 415 showed a five-fold increase of colon cancer risk in Israelites (Jews). However, similar studies by Shin et al. [12] and Schafmayer et al. [13] in other two populations did not find the similar conclusion. This suggests that further extensive studies in other populations are needed to resolve this controversy.

In our study, the frequency of genotype C/C of hMLH1 gene at site 415 was significantly higher in sporadic colon cancer patients than in controls in Chinese Han population (OR=5.29, 95%CI=1.07-26.04). This result is similar to Lipkin's finding (OR=5.0, 95%CI: 0.6-42.8) [11]. It is worth noting that the rate of G→C mutation in our samples (both patients and controls) is higher than Lipkin's samples. This suggests that the rate of G→C mutation at position 415 of hMLH1 gene could vary in different ethnic groups, supporting the notion that genetic backgrounds in different races also contributes to the complexities of the relationship between this mutation and susceptibility to colon cancer.

The mechanism underlying the increased susceptibility of colon cancer in people with hMLH1 415G→C mutation remains to be elucidated. While MSI due to dysfunction or mutation of MMR is a characteristic feature of hereditary colon cancer, it has been reported that MSI is not common in sporadic cancers [16, 17]. It is unclear whether MSI is present in a small group of sporadic colon patients with hMLH1 415G→C. Lipkin et al. [11] thought that the hMLH1 415G→C mutation prevents hMLH1 from combining with ATP, which impairs the process of DNA repair. Therefore, one possible mechanism is that this particular 415G→C mutation may result in MSI, which in turn increase the risk of colon cancer (18, 19). On the other hand, Rahner et al. [20] reported that this mutation led to a reduction in hMLH1 gene expression and a subsequent decrease of DNA repair. However, our results indicated that the hMLH1 mRNA expression has no difference between 415G→C mutation and other genotype groups. These differences among different studies may be due to variations of experimental design or ethnic populations. Further studies at both expression and function levels are necessary to understand the mechanism how this mutation is involved in the tumorgenesis of sporadic colon cancers.

Concerning the relationship between hMLH genotypes and clinical characteristics, we did not detect significant differences among different subgroups of patients who have been divided according to different clinical phases, pathological types, tumor size, or tumor locations ($p > 0.05$). These results may be due to two possible reasons. First, this mutation may be only relevant to the initiation of the tumorgenesis but not to the further development. Then, the frequency of the genotype will be correlated with the

susceptibility but not the clinical characteristics. On the other hands, the case number is small (n <10) when the patients are further divided into subgroups, which may prevent us from detecting the actually existing difference.

In conclusion, G→C mutation in hMLH1 gene at site 415 was associated with an increase of susceptibility of sporadic colon cancer. This mutation might represent a genetic factor in a small group of sporadic colon cancer in Chinese Han population. However, this mutation did not appear to change hMLH1 gene expression.

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