



***MLH1* promoter germline-methylation in selected probands of Chinese hereditary non-polyposis colorectal cancer families**

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Abstract

AIM: To detect the *MLH1* gene promoter germline-methylation in probands of Chinese hereditary non-polyposis colorectal cancer (HNPCC), and to evaluate the role of methylation in *MLH1* gene promoter and molecular genetics in screening for HNPCC.

METHODS: The promoter germline methylation of *MLH1* gene was detected by methylation-specific PCR (MSP) in 18 probands from unrelated HNPCC families with high microsatellite-instability (MSI-H) phenotype but without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. At the same time, 6 kindreds were collected with microsatellite-stability (MSS) phenotype but without germline mutations in *MSH2*, *MLH1* and *MSH6* genes as controls. The results of MSP were confirmed by clone sequencing. To ensure the reliability of the results, family H65 with nonsense germline mutation at c.2228C > A in *MSH2* gene was used as the negative

control and the cell line sw48 was used as the known positive control along with water as the blank control. Immunochemical staining of *MLH1* protein was performed with Envision two-step method in those patients with aberrant methylation to judge whether the status of *MLH1* gene methylation affects the expression of *MLH1* protein.

RESULTS: Five probands with *MLH1* gene promoter methylation were detected in 18 Chinese HNPCC families with MSI-H phenotype but without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. Two of the five probands from families H10 and H29 displayed exhaustive-methylation, fulfilling the Japanese criteria (JC) and the Amsterdam criteria (AC), respectively. The other 3 probands presented part-methylation fulfilling the AC. Of the 13 probands with unmethylation phenotype, 8 fulfilled the JC and the Bethesda guidelines (BG), 5 fulfilled the AC. The rate of aberrant methylation in *MLH1* gene in the AC group (22.2%, 4/18) was higher than that in the JC/BG groups (5.6%, 1/18) in all HNPCC families with MSI-H phenotype but without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. However, no proband with methylation in *MLH1* gene was found in the families with MSS phenotype and without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. No expression of *MLH1* protein was found in tumor tissues from two patients with exhaustive-methylation phenotype, whereas positive expression of *MLH1* protein was observed in tumor tissues from patients with partial methylation phenotype (excluding family H42 without tumor tissue), indicating that exhaustive-methylation of *MLH1* gene can cause defective expression of *MLH1* protein.

CONCLUSION: Methylation phenotype of *MLH1* gene is correlated with microsatellite phenotype of *MMR* genes, especially with MSI-H. Exhaustive-methylation of *MLH1* gene can silence the expression of *MLH1* protein. *MLH1* promoter methylation analysis is a promising tool for molecular genetics screening for HNPCC.

Key words: Hereditary non-polyposis colorectal cancer; *MLH1*; Methylation; Germline; Methylation-specific PCR; Microsatellite phenotype

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INTRODUCTION

Hereditary non-polyposis colorectal cancer (HNPCC), also known as Lynch syndrome, is characterized by an autosomal dominant inheritance of early-onset microsatellite instability (MSI)-positive colorectal cancer and an increased risk of other cancers, including cancers of the endometrium, stomach, ovary, urinary tract, pancreas, and small bowel. HNPCC accounts for 5%-10% of all colorectal cancers and is caused by a mutation in one of the following DNA mismatch repair (MMR) genes: *MLH1*, *MSH2*, *MSH6*, *PMS1* and *PMS2*^[1-3]. Germline mutations in *MLH1* and *MSH2* account for > 90% of all known MMR mutations in HNPCC^[4], and germline mutations in *MSH6* account for 5%-10%, whereas mutations in other genes are rare^[3,5]. MSI has been observed in approximately 13% of sporadic colorectal cancers (CRC) and in virtually all CRC arising in patients with HNPCC. Germline mutations in MMR genes, high-frequency microsatellite instability (MSI-H) and loss of MMR protein expression are the hallmarks of HNPCC. Epigenetic silencing is usually considered a kind of somatic phenomenon and somatic *MLH1* promoter hypermethylation is generally accepted in the tumorigenesis of sporadic tumours. However, little is known about the maintenance of epigenetic state in the germline^[6] and abnormal *MLH1* gene promoter methylation in normal body cells is controversially discussed as a mechanism predisposing patients to HNPCC. Recently, aberrant methylation in MMR genes, *MLH1* or *MSH2*, has been supposed as a basic factor for cancer^[7]. Promoter hypermethylation in *MLH1* gene of colorectal tumors correlates well with loss of MLH1 protein in sporadic MSI-positive cases^[8,9]. This study was to investigate the *MLH1* gene germline epimutation by methylation-specific PCR (MSP) in 18 Chinese HNPCC kindreds with MSI-H but without germline mutations in *MSH2*, *MLH1*, or *MSH6* gene, in order to identify HNPCC families and provide experimental information for HNPCC database.

MATERIALS AND METHODS

Materials

From January 1998 to October 2005, 24 Chinese HNPCC families fulfilling different clinical criteria were registered at the Department of Abdominal Surgery in Shanghai Cancer Hospital/Institution. Germline mutations in *MLH1*, *MSH2* and *MSH6* genes were excluded by DNA-PCR-based sequencing in the probands of all Chinese HNPCC families^[10-12]. Of them, 18 unrelated HNPCC probands were selected for the study objects

Table 1 Characteristics of 18 probands with MSI-H

Case	Gender	Age (yr)	Criteria	MSI	<i>MLH1/MSH2/MSH6</i> mutation study
H21	M	38	AC	MSI-H	NM
H22	M	46	AC	MSI-H	NM
H28	F	30	AC	MSI-H	NM
H29	F	37	AC	MSI-H	NM
H32	M	51	AC	MSI-H	NM
H42	M	65	AC	MSI-H	NM
H46	M	48	AC	MSI-H	NM
H57	F	47	AC II	MSI-H	NM
H63	F	47	AC	MSI-H	NM
H10	M	41	JC	MSI-H	NM
H12	F	50	JC	MSI-H	NM
H41	M	46	JC	MSI-H	NM
H55	M	49	JC	MSI-H	NM
H7	M	38	BG	MSI-H	NM
H8	M	43	BG	MSI-H	NM
H30	M	48	BG	MSI-H	NM
H35	F	38	BG	MSI-H	NM
H51	F	27	BG	MSI-H	NM

AC: Amsterdam criteria; JC: Japanese criteria; BG: Bethesda guidelines; MSI-H: High microsatellite instability; NM: No mutation.

Table 2 Characteristics of 6 probands with MSS

Case	Gender	Age (yr)	Criteria	MSI	<i>MLH1/MSH2/MSH6</i> mutation study
H16	F	44	JC	MSS	NM
H20	F	54	BG	MSS	NM
H44	F	39	BG	MSS	NM
H48	M	28	BG	MSS	NM
H50	M	55	BG	MSS	NM
H54	M	43	BG	MSS	NM

JC: Japanese criteria; BG: Bethesda guidelines; MSS: Microsatellite stability; NM: No mutation.

with the phenotype of MSI-H, and the remaining 6 were for the control group with the phenotype of microsatellite stability (MSS). Each participant was asked to give 10 microliters of peripheral blood and consented for access to archival tumor tissue. The characteristics of the selected cases are listed in Tables 1 and 2. To ensure the reliability of the results, family H65 with nonsense germline mutation at c.2228C > A in *MSH2* gene was used as the negative control and the cell line sw48 was used as the known positive control for the methylation in *MLH1* gene as well as water as the blank control. This study was proved by the Medical Ethical Committee of Cancer Hospital, Fudan University. The procedures of the study were in accordance with the international rules and regulations.

DNA extraction

Genomic DNA from peripheral blood and the cell line sw48 was extracted with the QIAGEN (Hilden, Germany) DNA extraction kit following its manufacturer's introductions. Concentration of the genomic DNA was determined with an ultraviolet spectrophotometer (Beckman, DU640 type).

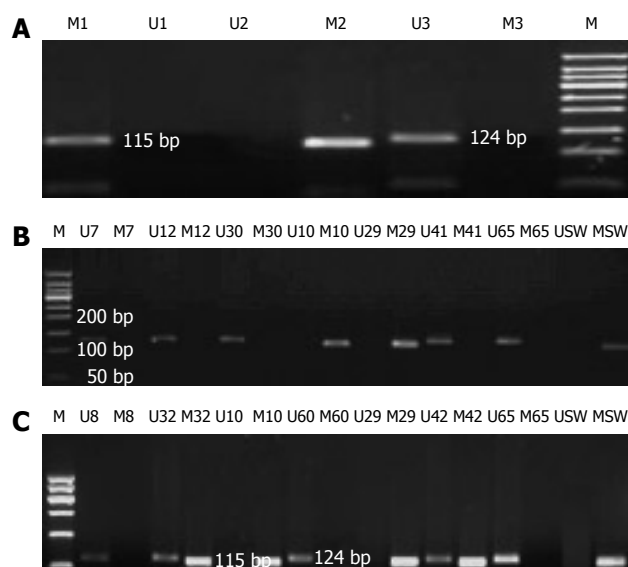


Figure 1 Results of *MLH1* MSP assay using primers that amplify methylated (M) or unmethylated (U) alleles (Lane M represents 100-bp DNA marker).
 A: *MLH1* MSP assay in families H10, H65 and cell line SW48. M1, M2, U1 and U2 indicate the methylated and unmethylated products of family H10, cell line SW48, and the methylated band (115 bp); M3 and U3 indicate the methylated and unmethylated products in family H65; B: *MLH1* MSP assay in families H7, H12, H30, H10, H29, H41, H65 and cell line SW48. U7, U12, U30, U41 and U65 indicate the unmethylated products of families H7, H12, H30, H41, and H65, respectively; M10, M29 and MSW indicate the methylated products of families H10, H29, and positive control SW48, respectively; C: *MLH1* MSP assay in families H8, H32, H10, H60, H29, H42 H65 and cell line SW48. U8, U60 and U65 indicate the unmethylated products of families H8, H60 and H6, respectively; M10, M29 and MSW indicate the methylated products of families H10, H29, and positive control SW48, respectively; U32, M32 and U42, M42 are products of families H32 and H42, respectively.

PCR for methylation in *MLH1*

MSP exploits the effect of sodium bisulfite on DNA, which efficiently converts unmethylated cytosine to uracil with methylated cytosine unchanged. Consequently, after treatment, methylated and unmethylated alleles have different sequences that can be used to design allele-specific primers.

Genomic DNA was modified with sodium bisulfite as described previously^[13,14]. The modified DNA was then subjected to MSP using primer pairs engineered to amplify either methylated or unmethylated DNA. Methylated and unmethylated primer pair sequences were also designed as previously described^[15] and synthesized (Sangon, Shanghai). PCR was carried out with HotstarTaq DNA polymerase (Cat. No. 203203): preheating at 94°C for 10 min, followed by 40 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 45 s and extension at 72°C for 45 s, and a final elongation at 72°C for 7 min. PCR products were subjected to 2% agarose gel electrophoresis. The products of exhaustive-methylation only indicated a methylated band of 124 bp and the unmethylated products only indicated an unmethylated band of 115 bp, while the partially methylated products indicated both of them. After observation of clear and expected bands, the products were purified using the QIAquick gel extraction kit (Qiagen) and sequenced on a 3700 DNA sequence system (ABI, USA) in order to check the correct bisulfite

modification. Appropriate positive and negative reference samples were included. Each result of sequencing was analyzed by DNA Star 5.08 bioanalysis software.

Immunochemical staining for *MLH1*

A monoclonal antibody against *MLH1* (Pharmingen, San Diego, CA, USA) was prepared at a 1:40 dilution and detected by the Envision two-step method to judge whether the status of methylation in *MLH1* gene would affect the expression of *MLH1* protein. The expression of *MLH1* was diminished in cancer tissues in the absence of detectable nuclear staining of neoplastic cells. Infiltrating lymphocytes and normal colonic crypt epithelium next to the tumor area served as internal positive controls.

RESULTS

Five probands with *MLH1* gene methylation were found in 18 unrelated Chinese HNPCC families with MSI-H phenotype but without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. The rate of abnormal methylation in *MLH1* gene was approximately 27.8% (5/18). Among the 18 patients, 2 displayed exhaustively methylated phenotype and the other 3 presented partially-methylated phenotype. The exhaustive methylation accounted for 11.1% (2/18) in the HNPCC families with MSI-H but without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. Perhaps, the changes might be much lower in all unselected HNPCC families. Among the 13 probands with unmethylation phenotype, 8 fulfilled the Japanese criteria (JC)/Bethesda guidelines (BG), 5 fulfilled the Amsterdam criteria (AC). All probands with partially-methylated phenotype fulfilled the AC, whereas probands of families H10 and H29 displaying exhaustively-methylated phenotype fulfilled the JC and AC, respectively. The rate of aberrant methylation in *MLH1* gene in the AC group (22.2%, 4/18) was higher than that in the JC/BG groups (5.6%, 1/18) in all HNPCC families with MSI-H phenotype and without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. However, no proband with methylation in *MLH1* gene was found in HNPCC families with MSS phenotype but without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. In our study, the expected size of bands was clear and specific. The study was repeated in triplicate to make sure all results credible (Figure 1A-C). Moreover, all exhaustively and partially methylated PCR products were purified and clone-sequenced in order to further substantiate the results of MSP (Figure 2). We believed that the methylation in *MLH1* gene might be related with microsatellite phenotype. No expression of *MLH1* protein was observed in tumor tissues from two patients with exhaustively methylated phenotype, while positive expression of *MLH1* protein was found in tumor tissues from patients with partially methylated phenotype (excluding family H42 without tumor tissue), suggesting that exhaustive-methylation in *MLH1* gene can cause defective expression of *MLH1* protein and influence its function while the partial methylation in *MLH1* gene may have no impact on the expression of *MLH1* protein.

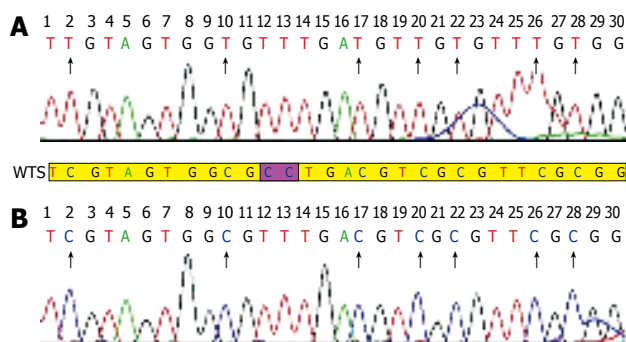


Figure 2 Methylation analysis of the promoter *MLH1* gene by clone sequencing. Arrow indicates CpG dinucleotide, WTS indicates the wild-type sequence of transcription start site. A: Unmethylated band presenting all unmethylated cytosines was converted to uracil after bisulfite modification; B: Methylated band presenting all unmethylated cytosines was unchanged after bisulfite modification.

DISCUSSION

HNPCC syndrome is the most common form of hereditary colorectal cancer. Predisposed individuals have a higher risk of developing cancer. The syndrome is primarily due to heterozygous germline mutations in *MLH1*, *MSH2*, *MSH6* and *PMS2* genes. The resulting mismatch repair deficiency leads to MSI which is the hallmark of tumors arising within this syndrome, as well as a variable proportion of sporadic tumors. Diagnostic guidelines and criteria for molecular testing of suspected families have been proposed and continuously updated. However, not all families fulfilling these criteria show mutations in MMR genes and/or MSI implicating other unknown carcinogenic mechanisms and predisposition genes. This subset of tumors is the focus of current clinical and molecular research.

Germline mutations in the coding regions of *MSH2* and *MLH1* genes are known to be responsible for up to 45%-64% of all HNPCC families^[16], and those of *MSH6* account for 10% of HNPCC kindreds^[17]. We have previously detected germline mutations in the entire coding regions of *MSH2*, *MLH1* and *MSH6* genes in 24 probands meeting the AC, 15 probands fulfilling the JC and 19 probands meeting the BG by PCR-gene-sequencing with 20 germline mutations detected including two mutations occurring in a same patient and three novel mutations^[10,11]. Subsequently, 3 new mutations are found by mRNA-based PCR sequencing^[12]. It was speculated that the remaining probands without mutations in *MSH2*, *MLH1*, and *MSH6* genes might be associated with other aberrant types of genes. It was reported that DNA methylation associated with transcriptional silencing of *MLH1* is the underlying cause of MMR defects in most sporadic colorectal cancers with a MSI+ phenotype^[9,18]. Moreover, reversal of methylation with 5-aza-deoxycytidine not only results in reexpression of MLH1 protein, but also restoration of the MMR capacity in MMR-deficient cell lines^[9]. These findings further substantiate that the promoter methylation in *MLH1* gene is another deficient mechanism of *MLH1* gene.

Hypermethylation of CpG island in the promoter se-

quence has been proved to be an important mechanism of gene silencing and is particularly associated with transcriptional silencing of tumor suppressor genes in sporadic cancers^[19,20]. Germline mutations might occur in individuals with a well-characterized genetic disease but lack an identifiable mutation in known disease genes^[21]. It was recently reported that monoallelic promoter hypermethylation in *MLH1* gene is observed in peripheral blood from a number of patients with early-onset colorectal cancer^[7,22-24]. The above results indicate that *MLH1* promoter-germline mutation might be related to HNPCC.

Our study demonstrated 5 probands with *MLH1* gene methylation (including 2 exhaustive-methylations which fulfill the JC and the AC, respectively, and 3 part-methylations fulfilling the AC) in 18 unrelated Chinese HNPCC families with MSI-H phenotype but without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. The rate of aberrant methylation in *MLH1* gene (22.2%, 4/18) was higher in probands fulfilling the AC than that (5.6%, 1/18) in those meeting the JC and BG. Of the 13 probands with unmethylated phenotype, 8 fulfilled the JC and BG (61.5%, 8/13), 5 fulfilled the AC (38.5%, 5/13). However, no proband was detected with the aberrant methylation in *MLH1* gene in the 6 suspected HNPCC families with MSS phenotype and without germline mutations in *MSH2*, *MLH1* and *MSH6* genes. These findings illuminate that the promoter methylation in *MLH1* gene is likely another underlying cause of MMR defect in HNPCC fulfilling the AC. In order to reveal whether the aberrant methylation in *MLH1* gene influences the expression of MLH1 protein, immunostaining of MLH1 protein was carried out in 5 probands with *MLH1* aberrant methylation in our study. No expression of MLH1 protein was found in 2 probands with exhaustively methylated phenotype, whereas positive expression of MLH1 protein was observed in 2 probands with partially methylated phenotype (excluding family H42 without tumor tissue) suggesting that exhaustive methylation in *MLH1* gene can cause defective expression of MLH1 protein and influence its function while partial methylation of *MLH1* gene may have no impact on the expression of *MLH1* gene, revealing that methylation in *MLH1* gene may be related with the microsatellite phenotype and influence the expression of MLH1 protein and its function, which is consistent with the reported findings in other studies^[8,9].

In neoplastic cells, stable allele-specific loss of transcription due to aberrant methylation in an unmutated promoter region can identify hypermethylation as a direct mechanism of tumor suppressor gene inactivation^[25]. Moreover, the promoter methylation can be passed in somatic mitosis, which is reversible. Persons with hypermethylation in *MLH1* alleles of somatic cells can predispose to the development of cancer in patterns with hereditary nonpolyposis colorectal cancer. It was reported that epimutation can be transmitted from a mother to her son^[26], which is consistent with transgenerational epigenetic inheritance.

In the present study, the rate of aberrant methyla-

tion in *MLH1* gene was only 27.8% (5/18) in selected HNPCC with MSI-H phenotype but without germline mutations in *MLH1*, *MSH2* and *MSH6* genes. Among the probands with aberrant methylation, the rate of methylation in those fulfilling the AC accounted for 80% (4/5), which was significantly higher than that [20% (1/5)] in those meeting the JC and BG. Methylation analysis of the *MLH1* promoter should be performed for all early-onset or multiple colorectal cancer patients with MSI-H tumors or loss of *MLH1* protein expression due to unknown causes in HNPCC probands fulfilling the AC.

There is evidence that aberrant methylation in the promoter region of *MLH1* alleles is functionally equivalent to a pathogenic *MLH1* germline mutation and mimics the clinical phenotype of Lynch syndrome. 'Sporadic' HNPCC-patients need to be treated Lynch syndrome patients. Individuals carrying *MLH1* germline epimutations are at a high risk of developing colorectal and other tumors and should be considered carriers of germline mutations. Inheritance should be discarded in each case, until more conclusive data are obtained. *MLH1* promoter methylation analysis should be performed at least for the first degree relatives with positive methylation to exclude the inheritance of a familial epimutation^[27]. Identification of hypermethylation as an epigenetic defect has important implications for surveillance recommendations, since these patients should be treated like Lynch syndrome patients. The heritability of methylation needs to be further investigated.

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COMMENTS

Background

Germline mutations in mismatched repair genes, such as *MLH1*, *MSH2* and *MSH6*, lead to hereditary nonpolyposis colorectal cancer (HNPCC) and not all families fulfilling these criteria show mutations in mismatched repair genes. It is well known that *MLH1* promoter methylation is related with sporadic colorectal cancer. However, *MLH1* promoter germline-methylation in Chinese HNPCC patients has not yet been reported.

Research frontiers

Germline mutations in MMR genes, such as *MSH2*, *MLH1* and *MSH6* contribute to the early diagnosis of HNPCC and screening of HNPCC families. Few studies on *MLH1* promoter germline-methylation are available.

Innovation and breakthroughs

Five patients with *MLH1* gene methylation were found in this study by methylation-specific PCR in 18 unrelated Chinese HNPCC probands with high microsatellite-instability phenotype but without germline mutations in *MSH2*, *MLH1* and *MSH6* gene. The rate of abnormal methylation in *MLH1* gene was approximately 27.8% (5/18) and the rate (22.2%, 4/18) in probands fulfilling the Amsterdam criteria, which was higher than that (5.6%, 1/18) in those meeting the Japanese criteria/Bethesda guidelines.

Applications

MLH1 promoter methylation analysis can be used for the microsatellite phenotype of mismatched repair genes and is a promising tool for molecular genetics screening of HNPCC.

Terminology

HNPCC is an abbreviation of hereditary nonpolyposis colorectal cancer; MSP is an abbreviation of methylation-specific PCR.

Peer review

In this study, *MLH1* promoter germline-methylation was detected in 18 unrelated Chinese HNPCC probands with high microsatellite-instability phenotype but without germline mutations in *MSH2*, *MLH1* and *MSH6* gene. The rate of aberrant methylation in probands meeting the Amsterdam criteria was higher than that in those fulfilling the Japanese criteria/Bethesda guidelines. However, the function of *MLH1* promoter germline-methylation should be further studied with a large of samples.

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