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

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

METHODS: The promoter germline methylation of **MLH1** gene was detected by methylation-specific PCR (MSP) in 18 probands from unrelated HNPPC 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. Immunohistochemical 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 HNPPC 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 HNPPC 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 HNPPC.

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[5,6]. 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[7] 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 tumours 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 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 QIAamp DNA extraction kit following its manufacturer’s introductions. Concentration of the genomic DNA was determined with an ultraviolet spectrophotometer (Beckman, DU640 type).

### Table 1 Characteristics of 18 probands with MSI-H

| Case | Gender | Age (yr) | Criteria | MSI | MLH1/MSH2/MSH6 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       | 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 | MLH1/MSH2/MSH6 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.
RESULTS

Five probands with \textit{MLH1} gene methylation were found in 18 unrelated Chinese HNPCC families with MSI-H phenotype but without germline mutations in \textit{MSH2}, \textit{MLH1} and \textit{MSH6} genes. The rate of abnormal methylation in \textit{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 \textit{MSH2}, \textit{MLH1} and \textit{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 \textit{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 \textit{MSH2}, \textit{MLH1} and \textit{MSH6} genes. However, no proband with methylation in \textit{MLH1} gene was found in HNPCC families with MSS phenotype but without germline mutations in \textit{MSH2}, \textit{MLH1} and \textit{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 \textit{MLH1} gene might be related with microsatellite phenotype. No expression of \textit{MLH1} protein was observed in tumor tissues from two patients with exhaustively methylated phenotype, while positive expression of \textit{MLH1} protein was found in tumor tissues from patients with partially methylated phenotype (excluding family H42 without tumor tissue), suggesting that exhaustive-methylation in \textit{MLH1} gene can cause defective expression of \textit{MLH1} protein and influence its function while the partial methylation in \textit{MLH1} gene may have no impact on the expression of \textit{MLH1} protein.
Hypermethylation of CpG island in the promoter region can identify hypermethylation as a direct mechanism of tumor suppressor gene inactivation, 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-methylation which fulfill the JC and the AC, respectively, and 3 partial-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 ravel 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 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\(^{[8,9]}\).

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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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