**Research Article**

**A Novel MLH1 Initiation Codon Mutation (c.3G>T) in a Large Chinese Lynch Syndrome Family with Different Onset Age and mRNA Expression Level**

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Lynch syndrome is a genetically and clinically heterogeneous disorder; it is caused by a germline mutation in DNA mismatch repair (MMR) genes. Individuals with a heterozygous mutation in MLH1 have an increased risk for developing colorectal cancer. Here we described a 5-generation Chinese Lynch syndrome family with different severity and onset age. A novel heterozygous germline mutation (c.3G>T, p.Met1Ile) in MLH1 gene was discovered by next generation sequencing. Our study also revealed by qPCR that the MLH1 mRNA expression in peripheral blood of patients in this family was remarkably lower than that of the unaffected carriers and non-carriers. The research results indicated that the mRNA expression level may provide predictive suggestions of treatment and management for carriers with the initiation codon mutation of MLH1 in this family. Further studies are undertaken in this family as well as other families with Lynch syndrome to interrogate the exact reasons affecting the MLH1 mRNA expression level and whether mRNA expression in peripheral blood could be a significant factor for early diagnosis and surveillance of Lynch syndrome.

1. Introduction

Lynch syndrome (LS), with an autosomal dominant mode of inheritance, is also known as hereditary nonpolyposis colorectal cancer (HNPCC) [OMIM#120435]. Some germline mutations in DNA mismatch repair (MMR) genes, such as MLH1, MSH2, MSH6, and PMS2, are associated with Lynch syndrome. MMR genes play an important role in recognizing and repairing erroneous insertions, deletions, and other mutations that occur during DNA replication [1]. There are several other genes, such as EPCAM, MLH3, and PMS1, that may contribute to the mismatch repair process; these genes have been reported in some families with Lynch syndrome [2–4]. Lynch syndrome is a genetically and clinically heterogeneous disorder. Different types of pathogenic variants in different MMR genes may result in different cumulative risk, clinical phenotypes, and onset age at diagnosis. The same mutation in a family may have a wide variety of clinical phenotypes; this potentially complicates surveillance, counseling, diagnosis, and treatment strategies for mutation carriers.

Hereditary nonpolyposis colorectal cancer-2 (HNPCC2) is caused by the germline mutation of MLH1 gene [5]. The cumulative risk of MLH1 associated colorectal cancer for men and women is 75.4% and 76.9%, respectively [6]. The average age of diagnosis in HNPCC2 kindreds is 38.5 years, which is earlier than other types of HNPCC [7]. The cumulative 5-year survival following colorectal cancer in MLH1 mutation carriers was 56.2% [8, 9]. A simple tool to predict the age of onset, severity, and survival rate of MLH1 mutation carriers would be of prime clinical importance.
Previous studies of Lynch syndrome mainly focused on molecular genetic screening of the germline MMR genes, immunohistochemistry (IHC), and microsatellite instability (MSI) of the tumor tissue. However, germline mutation only provides a general lifetime risk for MLH1 mutation carriers [6, 10]. The detection of tumor tissue can only be performed on cancer patients. There may be a distinct difference in severity and age of onset in this family which cannot be predicted by standard tests.

In this study, we investigate a Chinese family with Lynch syndrome for five generations. The patients of this family exhibited a significantly different onset age and severity. A novel nucleotide substitution (c.3G>T) in MLH1 gene was detected from this family. We studied the MLH1 mRNA level in the peripheral blood of the proband and his family members (including Lynch syndrome survivors) by real-time quantitative polymerase chain reaction (qPCR). Our goal was to search for a way to predict the manifestation and to provide individualized surveillance (such as routine colonoscopy and prophylactic removal) before the development of cancer for each MLH1 mutation carrier in this family.

2. Materials and Methods

2.1. Identification of the Novel MLH1 Mutation. Because of different severity and onset age in this family, we performed target exome-based next generation sequencing on DNA samples of the proband and his father in order to exclude compound heterozygous mutations in the MMR genes [11].

2.2. Targeted Exome Based High Throughput Sequencing. In order to discover all the possible pathogenic mutations related to colorectal cancer in this family, 14 CRC related genes, including all the MMR genes, were selected from OMIM (http://www.ncbi.nlm.nih.gov/omim/). Roche NimbleGen's (Madison, USA) custom Sequence Capture Human Array covering the exonic sequences (except for the high repetitive regions of STK11 exon1) and flanking 10bp was used. The library preparation was consistent with standard protocols. Enriched DNA samples were pooled and sequenced on Illumina HiSeq 2500 Analyzers (Illumina, San Diego, USA) to generate 90 bps of paired-end reads.

2.3. Variant Annotation and Interpretation. Illumina Pipeline software (version 1.3.4) was used to generate raw data. Clean reads were then generated from the raw reads following our filtering criteria. Sequence alignment of the clean data was performed using NextGENe software (SoftGenetics, State College, Pa). The reference was obtained from the NCBI, version GRCh37 (hg19). SNVs were called according to our previously reported method [12]. In order to detect large exonic deletions and duplications together, a coverage-based algorithm [13] was used in our interpretation.

2.4. Sanger Sequencing. Sanger sequencing was performed on the proband and his family members to verify the true positive of the identified mutation and to confirm the carriers. Primers designing, PCR amplification, and sequencing were carried out according to the standard protocols at www.impactjournals.com.

2.5. Real-Time Quantitative RT-PCR. We collected 5 mL peripheral blood samples from the proband, as well as 8 of his family members. These samples were stored in 10 mL vacuum tube containing 200 µl of 0.5mol/L EDTA. Ficoll-Hypaque density gradient centrifugation was used to extract human peripheral blood mononuclear cells (PBMCs). TRizol Reagent (Invitrogen) was used to extract total RNA from the PBMCs. The RNA concentration and purity were checked by OD A260/A280 (>1.8) and A260/A230 (>1.6). ABI High-Capacity cDNA reverse transcription kits were used to make reverse transcriptions of 2 µg of total RNA. Synthesized cDNA was then stored at -20°C for later use. Using GAPDH as a housekeeping gene, the PCR was performed in triplicate using ABI viii7 real-time PCR detection system (Applied Biosystems) [14]. The nucleotide sequences of primers and probes were as follows: MLH1, forward primer is 5'-CCCATTTGCACAGAG-3', and reverse primer is 5'-TTTTCAGAGCCAGCTTAC-3'; the forward primer of GAPDH is 5'-CTGCCAACGTGTACAGTG-3', and the reverse primer is 5'-TCAGTGTAGCCCAAGTGGC-3'. The relative MLH1 expression value was calculated via the 2−ΔΔCt method.

2.6. Mismatch Repair Protein Immunohistochemistry. Immunohistochemistry was performed on the proband's postoperative tissue in accordance with standard streptavidin–biotin–peroxidase procedures [15].

2.7. Statistics. All data were expressed as the mean ± SEM. Statistical comparisons were performed using Student's t-test (P values < 0.05 were considered to be statistically significant).

3. Results

3.1. The Family with Lynch Syndrome. Initially a 42-year-old male patient (III:1) was referred to the Second Department of Surgery, The Fourth Hospital of Hebei Medical University, and was clinically diagnosed with colorectal cancer. After a series of clinical detections of the proband and pedigree investigation of the 5-generation family (Figure 1), this family was diagnosed with Lynch syndrome according to Bethesda Guidelines which principally includes (1) colorectal cancer (CRC) diagnosed younger than 50 years, (2) CRC with the MSI-H histology, (3) one or more first-degree relatives diagnosed with a LS-related cancer before age of 50 years, and (4) at least one patient with CRC or other LS-related tumors diagnosed synchronously, or metachronously, regardless of age [16].

3.2. Family History and Clinical Description. In this 5-generation Chinese family, there are 3 family members clinically diagnosed with CRC (II:1, II:2, and III:4); 2 family members (I:2 and II:3) have died of intense abdominal pain at an early age without a timely diagnosis and treatment (Figure 1). They were supposed to die from colorectal cancer according to the
descriptions of their family members. However, there is not definite diagnosis or autopsy attributed to the poor medical condition decades before in the rural areas. The other 12 family members in Figure 1 have received general physical examinations with no other types of cancer associated with Lynch syndrome found.

3.3. II-2: The Proband’s Father. At the time of study, the proband’s father was 62 years old. He developed 2 colorectal carcinomas metachronously: one in his transverse colon at the age of 27 and the other in his descending colon at age of 58. After the second colectomy and partial resection of the ileum, he recovered.

3.4. III-3: The Proband’s Cousin. At the time of study, the proband’s elder cousin was 49 years old. She developed colorectal carcinomas at the age of 29. After experiencing intense abdominal pain, she was diagnosed with CRC. Early diagnosis and treatment enabled her to survive and recover.

3.5. III-1: The Proband. The proband is a 43-year-old man. He complained of occasional abdominal pain and blood in the stool for 5 years; this was accompanied by a defecation frequency of 5-6 times/day. These symptoms could be relieved after taking oral antiphlogistic medicine. However, aggravation of blood in stool compelled him to consult the physician. The colonoscopy identified 0.5 cm × 1.0 cm polyps with smooth mucosa in the transverse colon, as well as enormous ulcerative neoplasm with necrosis on the surface uplift at 35-40 cm away from the anal margin.

3.6. Sequencing Result. Only one novel initiation codon mutation (c.3G>T of MLH1 gene) was identified by the genetic screening from both the proband and his father. This mutation was verified by Sanger sequencing using primers: F-5'-AGACCCAGCAACCCACAG-3', R-5'-TTCTCCAC-TTACACTCCAAA-3'. According to the validation, this mutation was confirmed to be cosegregated in this family (Figures 1, 3(a), and 3(b)). This mutation leads to a nonfunctional MLH1 protein, which potentially reduces activity [17].

3.7. Real-Time Quantitative RT-PCR Result. The MLH1 mRNA level was measured from 1 normal control and 9 family members which includes the 3 patient carriers, 3 normal
Figure 2: The immunohistochemistry of the proband. (a) represents MSH2, (b) represents MSH6, (c) represents BRAF, (d) represents PMS2, and (e) represents MLH1.

Figure 3: (a) and (b) Sanger sequencing of MLH1 (c.3G>T, p.Met1Ile) in these family members identified in the proband and his father (GenBank Accession: NM_000249.3).
Table 1: The MLH1 mRNA expression level of in the family members.

| Family member | Mutation state | CRC state | MLH1 mRNA expression (2^{-ΔΔCt}) | Average expression (2^{-ΔΔCt}) |
|---------------|----------------|-----------|-----------------------------------|-------------------------------|
| III:1         | YES            | 38        | 0.8004/1.000/1.000               | 0.93347                       |
| II:2          | YES            | 27        | 0.7787/0.995/0.887               | 0.8692                       |
| III:3         | YES            | 29        | 0.7426/0.436/0.719               | 0.632556667                  |
| II:5          | YES            | no        | 2.1416/0.973/1.339               | 1.484533333                  |
| IV:1          | YES            | no        | 2.7604/1.957/2.606               | 2.441133333                  |
| IV:3          | YES            | no        | 9.9197/7.469/11.654              | 9.6809                       |
| IV:4          | YES            | no        | 1.2054/0.993/1.163               | 1.120466667                  |
| III:5         | NO             | no        | 1.5237/1.166/1.159               | 1.2829                       |
| II:1          | NO             | no        | 1.1539/1.635/1.304               | 1.3643                       |
| normal control| NO             | no        | 1/0.895/1.576                    | 1.157                        |

![Relative mRNA expression level](image)

**Figure 4:** The relative mRNA level of MLH1 in these family members with or without MLH1 mutation. III:1, II:2, and III:3 are patients, II:5, IV:1, and IV:4 are unaffected carriers, III:5 and II:1 are non-carriers in this family, and normal control is a non-carrier of MMRs gene with no blood relationship to this family. The levels of MLH1 in patients were lower than those with wild type MLH1 or unaffected carriers.

3.8. Statistics. We calculated the P value between the 3 patient carriers and the normal carriers (expect for IV:3 who is still so young, 9 years old, that his mRNA level might influence the average level) and between the 3 patient carriers and the 3 non-carriers, respectively (Figure 5). There is significant difference between the patient carriers and the normal carriers, as well as between the patient carriers and the non-carriers, respectively.

4. Discussion

The MLH1 initiation codon mutation (c.3G>T, p.Met1Ile) is a unique and potentially pathogenic MMR mutation detected in this family with Lynch syndrome and is cosegregated among the affected members in this family. It is classified with strong evidence of pathogenicity by American College of Medical Genetics and Genomics (ACMG) standards and guidelines [17]. Some other initiation codon mutations of the MLH1 gene have been reported in Lynch syndrome families. In vitro functional studies of MLH1 (c.1A>G, p.Met1Val) mutation revealed that the translation is mostly initiated 103 nucleotides downstream, but also at other two ATG sequences downstream. Because this mutation results in a frameshift mutation of start codon, it will lead to formation of truncated proteins. The major transcriptional product showed decreased mismatch repair activity in vitro compared to known pathogenic mutations. The other two transcripts showed either minimal protein expression (initiated at c.89) or moderate expression (initiated at c.122) (Parsons et al., 2015). Other initiation codon mutation of MLH1, (c.2T>A, p.Met1Lys) [18], (c.2T>C, p.Met1Thr) [19], and (c.2T>G, p.Met1Arg) [20, 21], were reported to be pathogenic because...
of MSI-H and abnormal IHC detected in tumors. The MLHI (c.3G>A, p.Met1Ile) mutation was detected in a family with Lynch syndrome [22]. This research provides more evidence of pathogenicity for this MLHI (c.3G>T, p.Met1Ile) mutation. Considering this family's pedigree, we speculate that this start codon mutation of MLHI is potentially pathogenic in nature; the partial loss of MLHI mRNA expression indicates that this mutation might be associated with an intermediate penetrance of the Lynch syndrome phenotype.

Recent research of MLHI mRNA levels in peripheral blood lymphocytes has concentrated on differentiating aspects of hereditary nonpolyposis colorectal cancer from normal controls [23], or determining mutation pathogenicity [24]. Controls in those studies are non-carriers of MMRs mutation. In our present research, we discovered that the carriers of the same MLHI mutation showed different mRNA levels in peripheral blood compared to the control group and carriers of the mutation that have not been affected by Lynch syndrome. The mRNA level of MLHI in carriers that have recovered from colorectal cancer decades ago is still lower than that of unaffected carriers and non-affected mutation carriers. As unaffected carriers have never suffered from HNPPC-related symptoms, the MLHI mRNA level is almost 2 times higher than the mutation carriers affected with Lynch syndrome. It should be noted that the relative gene expression of the 9-year-old unaffected carrier IV:3, which is not mentioned in Figure 3, is 12 times more than that of affected carriers. The relative gene expression of unaffected carrier IV:4, who is 27 years old, is lower than the average of the unaffected carriers and non-carriers; close follow-up will be taken on this carrier to make an early diagnosis. No significant relationships were found between mRNA levels in peripheral blood lymphocytes and the early diagnosis of the MMRs mutation carriers; besides the selective transcription of the initiation mutation, there are several other mechanisms which may also influence the expression of MLHI in different carriers. Epigenetic regulation may play crucial roles to drive carcinogenesis. Promoter methylation of MMRs could lead to reduced protein expression [25]. Histone modifications could regulate the hMLHI alternative splicing [26]. Some microRNA such as miR-422a could suppress MLHI expression through base paring with the MLHI 3’-untranslated region. A feedback loop could be formed between the microRNA transcription and the MLHI expression and controls cell growth and proliferation [27].

5. Conclusions

The detection of germline MMRs mutation, protein expression, and MSI [15, 28, 29] is mature and has been applied extensively in clinics. The MMRs mutation could help genetic counselors to predict the lifetime cancer risk of the carriers. The protein expression and MSI of tumor tissues could help doctors to evaluate the survival time as well as enable them to select suitable therapy. The initial purpose of genetic testing and immunohistochemistry was to determine an operation plan and to select appropriate chemotherapy. The carrier situation in this family provides more evidence to the intermediate penetrance of the detected MLHI (c.3G>T, p.Met1Ile) mutation. The mRNA expression level of MLHI in the peripheral blood of the family members suggests that it may serve as a biomarker for early diagnosis of Lynch syndrome. In order to determine whether the mRNA level of MMR is normal, the definition of normal range of the mRNA level of MMR depends on long-term follow-up of the unaffected carriers of this family and detection of large scales of similar Lynch syndrome families. Normal controls are also certainly required. Whenever the mRNA level of some unaffected carrier is obviously decreasing, the relative surveillance and detection should be applied. Our research provides a new avenue for a simple noninvasive technique in the early diagnosis of Lynch syndrome. We are planning to expand this study to a larger population to validate whether the mRNA level of MMR in peripheral blood detected by qPCR could be applied to large Chinese cohort.

Data Availability

The datasets generated and/or analyzed during the current study are not publicly available due to the Chinese ethic rules, but are available from the corresponding author on reasonable request.

Ethical Approval

This study was performed in compliance with the Helsinki declaration. The institutional review board on bioethics and biosafety (IRB) of BGI approved this study, and the IRB approval number is BGI-IRB 17196.

Consent

All the patients and family members participating in this project have given permission and have signed informed consent for the genetic testing.

Disclosure

There is no funding for this research. The patients paid for their own targeted exome based high throughput sequencing and the corresponding author paid for the detection of other family members and the research of this work.

Conflicts of Interest

The authors declare that they have no financial conflicts of interest, or anything else that may impede this research and its publication.

Authors’ Contributions

Zhongxin Li, Yanni Zhang, Huishuang Chen, and Santasree Banerjee designed this study. Huishuang Chen, Zhiyu Peng, and Ru Bai contributed to the molecular diagnosis analysis based on NGS. Zhongxin Li, Zhaolong Zhao, Jian Lv, Jianbin Sun, and Yanni Zhang worked on the clinical study. Wei Li contributed to the statistics analysis of this work. Huishuang...
Chen, Yanni Zhang, and Zhiyu Peng wrote the paper. Huishuang Chen, Keke Lin, and Hui Huang contributed to the picture plots. Yanni Zhang, Huishuang Chen, and Zhiyu Peng made equal contributions. All authors read and approved the final manuscript and consent to publish this case report.

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