PO Box 2345, Beijing 100023, China
www.wjgnet.com
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RAPID COMMUNICATION

Clinical and molecular analysis of hereditary non-polyposis colorectal cancer in Chinese colorectal cancer patients

Jun Wang, Mao-Hong Luo, Zuo-Xing Zhang, Pei-Da Zhang, Xi-Li Jiang, Dong-Wang Ma, Rong-Zeng Suo, Li-Zhong Zhao, Qing-Hui Qi

Jun Wang, Hereditary Colorectal Tumors Registry and Tianjin Binjiang Hospital, Tianjin 300022, China
Mao-Hong Luo, Hereditary Colorectal Tumors Registry, Tianjin, China and Tianjin Medical University, Tianjin 300022, China
Zuo-Xing Zhang, Pei-Da Zhang, Xi-Li Jiang, Dong-Wang MA, Rong-Zeng Suo, Li-Zhong Zhao, Tianjin Binjiang Hospital, Tianjin 300022, China
Qing-Hui Qi, Tianjin Medical University Hospital, Tianjin, China
Supported in part by Tianjin Science Grant, China
Correspondence to: Dr. Jun Wang, Hereditary Colorectal Tumors Registry, Binjiang Road 205, Heping district, Tianjin 300022, China. chinahcc@vip.sina.com
Telephone: +86-22-27111390 Fax: +86-22-27121297
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Abstract

AIM: To analyze the frequency of hereditary non-polyposis colorectal cancer (HNPCC) in Chinese colorectal cancer (CRC) patients, and to discuss the value of microsatellite instability (MSI) and/or immunohistochemistry (IHC) for MSH2/MLH1 protein analysis as pre-screening tests in China.

METHODS: The Amsterdam criteria Ⅰ and Ⅱ (clinical diagnosis) and/or germline hMLH1/hMSH2 mutations (genetic diagnosis) were used to classify HNPCC families. Genetic tests, including microsatellite instability, immunohistochemistry for MSH2/MLH1 proteins and hMSH2/hMLH1 genes, were performed in each proband.

RESULTS: From July 2000 to June 2004, 1988 patients with colorectal cancer were analysed and 114 CRC patients (5.7%) from 48 families were categorized as having HNPCC, including 76 from 26 families diagnosed clinically and 38 from the other 22 families diagnosed genetically. The sensitivity and specificity of high MSI and IHC for predicting mutations were 100% and 54%, and 79% and 77%, respectively.

CONCLUSION: The frequency of HNPCC is approximately 10% among all Chinese CRC cases. The MSI and IHC detections for hMSH2/hMLH1 proteins are reliable pre-screening tests for hMLH1/hMSH2 germline mutations in families suspected of having HNPCC.

Key words: Hereditary non-polyposis colorectal cancer; Colorectal cancer; Mismatch repair gene; Immunohistochemistry; Microsatellite instability

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INTRODUCTION

In 1966, Henry Lynch and his colleagues described familial aggregation of colorectal cancer with stomach and endometrial tumors in two extended kindreds and termed it Cancer Family Syndrome[1], and then designated this constellation as "Lynch syndrome"[2]. In 1991, this condition was renamed "hereditary non-polyposis colorectal cancer (HNPCC)" by the International Collaborative Group on HNPCC (ICG-HNPCC), and the Amsterdam diagnosis criteria were published (Amsterdam criteria Ⅰ)[3]. In 1993, 1996 and 1997, the modified Amsterdam criteria[4,5], the Japanese criteria[6] and the Bethesda guidelines[7] were developed respectively for different purposes. In 1999, ICG-HNPCC proposed its own modification of the Amsterdam criteria Ⅰ, and termed it the Amsterdam criteria Ⅱ[8].

Since 1993, HNPCC has been confirmed to be associated with germline mutations in several DNA mismatch repair genes (MMR), including hMSH2, hMLH1, hMSH6, hPMS1, and hPMS2 (hMSH2 and hMLH1 mutations are the most common)[9-17]. The elucidation of the mismatch repair genes has added more diagnostic complexity[18-20]. The tumor tissue from HNPCC patients harbouring pathogenic mutations in the MMR genes is frequently characterized by microsatellite instability (MSI). Actually, carriers of pathogenic hMLH1/hMSH2 germline mutations show high MSI (H-MSI) in tumor tissues[21,22]. MSI and/or IHC analysis has been used as pre-screening tests to select individuals eligible for DNA mutation analysis in blood, and sequence analysis can avoid unnecessary, expensive and time-consuming DNA analyses[23].

Hereditary colorectal tumors registry, Tianjin, China was established in 1998. This study was conducted

to analyze the frequency of HNPCC among Chinese patients with colorectal cancer, and to discuss the value of microsatellite instability (MSI) and/or immunohistochemistry (IHC) for MSH2/MLH1 protein analysis as pre-screening tests in Chinese patients.

MATERIALS AND METHODS

Subjects

All colorectal cancer patients from the In-patient Department, Tianjin Binjiang Hospital and Tianjin Medical University Hospital seen between July 2000 and June 2004 were identified prospectively and included in the study. The inclusion criteria were: (1) patients with pathologically identified colorectal cancer, (2) self-reported Chinese born and living in mainland China, and (3) patients who agreed to participate in this study. We chose to categorise HNPCC patients using the following criteria: (1) patients from families meeting the Amsterdam criteria Ⅰ or Ⅱ and/or (2) patients who met less strict criteria for suspected HNPCC, but with pathogenic hMLH1/hMSH2 germline mutations. The suspected HNPCC was established in this study using the Japanese criteria and the top six guidelines of the Bethesda guidelines (the 7th guideline for young colorectal adenoma patients diagnosed before 40 years of age was not used in this study).

Each newly diagnosed HNPCC family was referred to Hereditary Colorectal Tumors Registry, Tianjin, China for the genealogic study, genetic counselling and DNA test in order to identify all the individuals of the family at risk and to ensure a lifelong follow-up.

Questionnaire

Surgeons completed a questionnaire covering malignancies and age at onset of cancer in the colorectal cancer patients and their first-degree relatives.

Genetic tests

hMSH2/hMLH1 germline mutation detection: Only HNPCC proband patients and suspected HNPCC proband patients underwent this test. Genomic DNA was isolated from peripheral blood using a kit from Dingguo Technologies. The 35 coding exons of hMLH1 and hMSH2 were amplified from purified DNA for SSCP analysis. The primers included part of the introns to detect possible splice mutations. In order to keep the length of the polymerase chain reaction (PCR) products for SSCP below 300 base pairs, hMLH1 exon 12, and hMSH2 exons 3, 12, and 14 were divided into two overlapping segments, giving a total of 39 PCR products. The PCR products were kept at -20℃ until SSCP analysis which was performed at a fixed gel temperature of 20℃. Sequencing was performed on PCR products with BigDye Terminator Cycle Sequencing Ready Reaction kits (Perkin Elmer) using standard conditions and the same sequencing primers as used for PCR. The PCR products were sequenced in both sense and antisense directions. SSCP and sequencing were performed using an ABI prism 377 sequencer, and analysed using the software programs GeneScan and Sequence Navigator (PE Applied Biosystems).

Microsatellite instability: Only HNPCC proband patients and suspected HNPCC proband patients underwent this test. MSI was determined by PCR of genomic DNA isolated from formalin-fixed, paraffin-embedded normal and colorectal cancer tissues from each proband. Tissue sections were deparaffinized in xylene, digested with proteinase K overnight at 55℃, and DNA was isolated using DNA kit (Dingguo Technologies, Beijing, China). PCR was carried out in 10 μL reactions containing 1 × manufacturer's PCR buffer, 1.5 mmol $MgCl_2$, 200 μmol/L deoxynucleotide triphosphates, 0.5 units of Taq polymerase (Dingguo Technologies), and 0.5 μmol/L of each primer. The forward primer was end-labeled using γ33P-ATP and polynucleotide kinase. The cycles were as follows: 8 min at 94℃, then 30 cycles of 94℃ for 30 s, 55℃ for 30 s and 72℃ for 30 s, and a final extension at 72℃ for 10 min. The products were subjected to electrophoresis in 2% denaturing polyacrylamide gels, which were subsequently dried and exposed to X-ray film (Kodak, BioMax) at room temperature overnight. To determine the extent of MSI, nine microsatellite markers were examined (BAT25, BAT26, BAT40, D2S123, D18S58, D10S197, D5S36, D18S69, and MYCL). Microsatellite was found to be unstable if one or more novel bands were present in the PCR product of the tumor sample as compared with the PCR product of normal tissues from the same individual. A tumor was considered to be H-MSI if 4 or more of the markers were unstable, L-MSI if fewer than 4 of the markers were unstable, and MSS if none of the nine markers was unstable.

Immunohistochemistry for MLH2/MSH1 proteins: HNPCC proband patients and suspected HNPCC proband patients underwent this test. Mouse anti-human monoclonal antibodies were used for the detection of mismatch repair proteins MSH2 (Sigma, Saint Louis, Missouri, USA) and MLH1 (EMD Biosciences, San Diego, CA, USA). Freshly cut paraffin sections were dewaxed in xylene, rehydrated in graded alcohols, and washed in TRIS buffer. Heat-induced epitope retrieval (HIER; 600 W microwaves twice for 15 min in pre-warmed 10 mmol/L sodium citrate buffer, pH 6) was employed for MSH2 and MLH1 staining. Primary antibodies were added (dilution: MSH2 1:50; MLH1 1:75) and slides were incubated overnight at 4℃. Slides were then processed on an immunostainer. Antigen antibody binding was visualized by the avidin-biotin-complex method using 3-amino-9-ethylcarbazole as a chromogen. The first antibody was replaced by phosphate-buffered saline as a negative control to assess the specificity of the antibodies. Haematoxylin-counterstained sections were mounted in aqueous mounting medium and observed under light microscopy. Surrounding normal colonic mucosa, stromal cells, and lymphocytes served as internal controls. Tumors were considered not to express either protein when the nuclei showed no immunostaining, but internal controls were positive.

Statistical analysis

SPSS Release 11.5 software was used for all analyses. The clinical diagnosis of HNPCC was established using each

Table 1 Diagnosis criteria of hereditary non-polyposis colorectal cancer

Amsterdam criteria Ⅰ

There should be at least three relatives with histologically verified CRC; and all the following criteria should be met:

1) One should be a first degree relative of the other two;
2) At least two successive generations should be affected;
3) At least one CRC should be diagnosed before age 50;
4) FAP should be excluded in CRC cases;
5) Tumors should be verified by pathological examination.

Amsterdam criteria Ⅱ

There should be at least three relatives with an HNPCC-associated cancer (CRC, cancer of the endometrium, small bowel, ureter or renal pelvis); and all the following criteria should be met:

1) One should be a first-degree relative of the other two;
2) At least two successive generations should be affected;
3) At least one cancer should be diagnosed before age 50;
4) FAP should be excluded in CRC cases (if present)
5) Tumors should be verified pathologically.

Japanese criteria

1 A case with 3 or more colorectal cancers within the first-degree relatives
2 A case with 2 or more colorectal cancers within the first-degree relatives meeting the following criteria:
a) Age of onset of colorectal cancer(s) being lower than 50;
b) With right colon involvement;
c) With synchronous or metachronous multiple colorectal cancers;
d) Associated with synchronous or metachronous extracolorectal malignancies.

Bethesda Guidelines

1 Individuals from families that fulfil the Amsterdam criteria;
2 Individuals with two HNPCC-related cancers, including synchronous or metachronous cancers;
3 Individuals with colorectal cancer, plus colorectal cancer and/or HNPCC-related cancer and/or colorectal adenoma in a first-degree relative; at least one of the cancers diagnosed before 45 yr of age and the adenoma diagnosed before age 40;
4 Individuals with colorectal or endometrial cancer diagnosed before age 45;
5 Individuals with right-sided colorectal cancer with an undifferentiated histopathological pattern (solid/cribiform) diagnosed before age 45;
6 Individuals with signet-ring cell type colorectal cancer diagnosed before age 45;
[1]7 Individuals with colorectal adenomas diagnosed before age 40.

[1]Condition 7 was not included in this study.

of the four defined criteria, and the detections of MSI and IHC were used as tests in two by two contingency tables to determine the sensitivity and the specificity. We evaluated the strength and weakness of each criterion by analyzing the clinical characteristics of the families with hMSH2 and hMLH1 mutations that were identified and missed by each of the respective criteria, and the same with detection of MSI and IHC. *P* values less than or equal to 0.05 were considered significant.

RESULTS

A total of 1988 patients with colorectal cancer were identified. Clinically and genetically diagnosed HNPCC patients and families are shown in Table 1 and 2.

One hundred and fourteen CRC patients (5.7%) from 48 families were categorized as having HNPCC, including: 76 clinically diagnosed HNPCC patients from 26 families (9 families with the Amsterdam criteria Ⅰ, 21 families with the Amsterdam criteria Ⅱ and 4 families with both) and 38 genetically diagnosed HNPCC patients from the other 22 families (all were clinically suspected HNPCC).

We found 391 clinically suspected HNPCC patients in 150 families in this study, including 116 colorectal cancer patients from 65 families, who fulfilled the Japan criteria, and 378 colorectal cancer patients from 145 families who fulfilled at least one of the top six Bethesda guidelines for the identification of HNPCC (Table 2).

Table 2 Number and size of families classified according to different clinical criteria for hereditary non-polyposis colorectal cancer

Clinical criteria	No. of families	Family size	Mean variance
Amsterdam I	9	15.2	37.2
Amsterdam II	21	14.3	20.5
Japanese criteria	65	6.7	13.2
Set A	26	8.7	9.3
Set B	61	6.9	10.6
Bethesda	145	8.3	9.6
Condition 1	9	15.2	37.2
Condition 2	58	13.6	20.7
Condition 3	89	11.7	8.9
Condition 4	105	10.9	7.6
Condition 5	9	7.2	8.5
Condition 6	2	9.9	12.6

In this study, 105 cases were diagnosed under 45 years of age, 29 cases (28%) were categorized as having HNPCC, including 12 clinically diagnosed HNPCC patients and 17 genetically diagnosed HNPCC patients.

hMSH2/hMLH1 germline mutation

In 26 clinically diagnosed HNPCC proband patients, 50% (13/26) showed germline mutation of hMSH2 or hMLH1 and 15% clinically suspected HNPCC proband

patients had positive results.

Microsatellite instability

Table 3 shows the results in each kind of families. The H-MSI phenotype was demonstrated in all 48 hMSH2/hMLH1 mutation carriers, and its sensitivity to mutations was 100%, and specificity was 54%.

Immunohistochemistry (IHC) for MLH2/MSH1 proteins

Among the 176 proband patients, 68 showed loss of staining for either MLH1 (28 cases) or MSH2 (40 cases). No case showed absence of both MLH1 and MSH2. For MLH2/MSH1 mutations, its sensitivity and specificity were 79% and 77%, respectively .

DISCUSSION

Diagnosis criteria

The diagnosis criteria of HNPCC are controversial due to the variety of clinical phenotypes associated with the syndrome in different areas or countries[4,5,7,30]. The International Collaborative Group on HNPCC established the Amsterdam criteria Ⅰ to provide a basis for uniformity in collaborative studies[3]. The criteria were restrictive, since extracolonic malignancies were not considered, and small families were unlikely to fulfil the criteria[31-33]. In this study, less than 19% patients were classified as the Amsterdam Ⅰ families. However, when Amsterdam Ⅰ criteria were met, the chance of HNPCC was high as it aimed at specificity rather than sensitivity. The Amsterdam criteria Ⅱ were also developed by the International Collaborative Group on HNPCC[4], and besides colorectal cancer, extracolonic malignant tumors were also included, such as endometrial, small bowel, ureter, renal pelvis cancers and other HNPCC-related malignant tumors, but the requirements were kept to three cases in at least two successive generations in the families. And small families were also unlikely to fulfil the second criteria. In this study, about 44% families were classified according to the Amsterdam criteria Ⅱ.

As the two Amsterdam criteria were considered to be too strict, the alternatives have been developed, such as the modified Amsterdam criteria required only two diagnosed relatives, and considered early-onset endometrial cancer or unusually early-onset neoplasm, and one of its two sets does not mention the number of generations[4,5]. The Japanese criteria did not mention the number of generations and also classified families with two cases, and additionally take into account the clinical features of HNPCC (such as early onset, multiple synchronous or metachronous colorectal cancers, and right colon involvement)[30] and 65 families were classified in this study. The Bethesda guidelines targeted the evaluation of colorectal tumors for microsatellite instability or mismatch repair gene testing, which are less restrictive and more sensitive, but less specific than the Amsterdam criteria[7], and 145 families were classified in this study.

The elucidation of the mismatch repair genes which are the genetic basis for many HNPCC families, has added more complexity[18,34,35].

Table 3 Microsatellite instability of HNPCC and suspected HNPCC probands *n* (%)

	Amsterdam Ⅰ (9 probands)	Amsterdam Ⅱ (21 probands)	Japanese (65 probands)	Bethesda (145 probands)
MSI-H	67 (6)	71 (15)	71 (46)	68 (99)
MSI-L	3 (3)	19 (4)	14 (9)	10 (14)
MSS	0 (0)	10 (2)	15 (10)	22 (32)

There was no significant difference among all these four kinds of patients.

HNPCC frequency in CRC

In China, this is the first population-based prospective study on a large series of consecutive CRC patients (n = 1988) whose family histories of malignant tumors were obtained from 114 HNPCC patients (5.7% of CRC patients) in 48 families and 105 patients diagnosed before 45 years of age, and 29 (28%) HNPCC patients.

Up to now, several study groups have estimated the frequency of HNPCC among consecutive CRC patients based on family histories without testing for hMLH1/hMSH2 germline mutations in Western countries. But estimation of the frequency of HNPCC based entirely on the Amsterdam criteria Ⅰ shows different results in different populations even in Western countries, ranging from 0.3% (Finnish) to 4.5% (Northern Italian)[25-27], even in the same Italian population, researches showed different frequency between 0.5% to 4.5%[27,28]. And 1.3% was found in this study. Differences in the frequency of HNPCC between populations may reflect the actual population differences but may also reflect differences in diagnoses among family members and differences in the level of proof that is accepted to verify diagnosis. Another approach to diagnose HNPCC was adopted by screening for germline mutations in hMLH1 or hMSH2. A Danish study group estimated the HNPCC frequency among 1200 consecutive colorectal cancer patients based on family histories and/or testing for hMLH1/hMSH2 germline mutations, and approximately 1.7% cases were diagnosed with HNPCC[29] which was much lower than our study (10%). And in our study, about 46% families with hMLH1 and hMSH2 germline mutations did not fulfill the two Amsterdam criteria, however only 2 of 20 families (10%) were found in the Danish study.

MSI and immunohistochemistry for MSH2/MLH1

MSI analysis was first described in 1993[36-38]. A H-MSI phenotype is reported in 85%-92% of colorectal carcinoma associated with HNPCC and in 10%-15% of sporadic CRC[21,39-42]. MSI analysis has a sensitivity of around 90% in detecting MMR deficiency in carriers of a pathogenic MMR mutation[43,44]. Several laboratories have reported H-MSI in all MSI- analysed patients with pathogenic hMLH1/hMSH2 mutations[21,22,45,46]. These results are in agreement with our findings as all patients with hMLH1/hMSH2 mutations in our study had H-MSI tumors using our panel of markers.

Immunohistochemical staining for the presence of the mismatch repair proteins offers a cheaper and simpler test,

and it is sensitive in predicting a truncating MMR defect in one of the genes[44,47]. IHC has additional advantages when compared with MSI analysis, indicating that the MMR gene is most eligible for DNA analysis. Since the mismatch repair proteins form heterodimeric complexes, distinct IHC patterns can be expected. Individuals can be selected for DNA mutation analysis, and the assessment of which gene to test first can be made. Previous studies have shown a high sensitivity in predicting mutations in MSH2 (92%) by applying IHC in colorectal tumors, but a lower sensitivity in MLH1 (48%)[44,45]. We got the likely results in this study.

Based on the results of our study, we propose that besides the two Amsterdam criteria, the Japan criteria and the Bethesda guidelines should also be used in clinical practice as the most inclusive clinical criteria for the diagnosis of HNPCC and genetic analysis should also be considered, and the detection of MSI and IHC for hMSH2 or hMLH1 protein is a reliable pre-screening test for hMLH1/hMSH2 mutations in families suspected of having HNPCC.

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