

• BRIEF REPORTS •

Clinicopathological and molecular genetic analysis of HNPCC in China

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14 (codon 743) of hMSH2 and a TTC deletion in exon 14 (codon 530) of hMLH1.

CONCLUSION: Chinese HNPCC have specific clinicopathological features, such as early onset, propensity to involve the proximal colon, and high frequency of multiple CRCs, liver cancer more frequent than endometrial cancer. Chinese HNPCC showed relatively frequent germline mutation of mismatch repair (MMR) genes that correlated closely with high-level MSI and loss of expression of MMR genes protein.

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Key words: Colorectal neoplasms; Hereditary nonpolyposis; Sequence analysis; Microsatellite instability; Mutation; Immunohistochemistry

Abstract

AIM: To explore the clinicopathological and molecular genetic features of hereditary nonpolyposis colorectal cancer (HNPCC) in Chinese population.

METHODS: We collected 16 Chinese HNPCC families from Wenzhou, Zhejiang Province, China. Tumor tissues and peripheral white blood cells were studied using microdissection, microsatellite analysis, immunostaining of hMSH2 and hMLH1 proteins and direct DNA sequencing of hMSH2 and hMLH1 genes.

RESULTS: (1) A total of 50 patients had CRC. Average age at diagnosis of the first CRC was 45.7 years; 40.9% and 28.7% of the CRCs were located proximal to the splenic flexure and in the rectum, respectively. Thirty-eight percent of the colorectal cancer patients had synchronous and metachronous CRC. 34.4% and 25% of the CRCs were poor differentiation cancer and mucinous adenocarcinoma, respectively. Fourteen extracolonic tumors were found, and the hepatic cancer was the most common tumor type. Twenty-one patients whose median survival time was 5.7 years died during 1-23 years. Twenty-nine patients have survived for 1-28 years, 58.6%, 41.4% and 24.1% patients have survived for more than 5, 10 and 15 years, respectively; (2) All nine tumor-tissues showed microsatellite instability (MSI) at more than two loci. Four tumor-tissues lost hMSH2 protein expression and one lost hMLH1 protein expression. Three pathological germline mutations were identified from five genetically analyzed families; two of three mutations had not been reported previously as they were a transition from C to A in exon

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INTRODUCTION

In 1895, Warthin first described some families with an excess of colorectal, uterine and gastric cancers. In 1960s, Lynch accurately described these cancer-prone families. This condition was first termed the "cancer family syndrome" and was later renamed hereditary nonpolyposis colorectal cancer (HNPCC). According to the absence or presence of extracolonic malignancies, these families were divided into Lynch syndrome I (hereditary site-specific colorectal cancer) and Lynch II syndrome (colorectal cancer in association with extracolonic cancer)^[1]; which accounts for 1-10% of the total colorectal cancer population^[1-5]. Clinically, it is diagnosed by Amsterdam criteria^[6]: (1) three or more relatives with histologically verified colorectal cancer, one of whom is the first-degree to the other two; (2) colorectal cancer affecting at least two generations; and (3) one or more colorectal cancer cases diagnosed before the age of 50. In addition, familial adenomatous polyposis (FAP) must be ruled out. As the criteria are too rigid for small families, they exclude extra-colonic cancers associated with HNPCC. In Asia, on the other hand, the Japan Research Society for Cancer of the Colon and Rectum developed the clinical criteria (Japanese criteria) for HNPCC in 1991^[7]. The criteria

include A: a case with three or more colorectal cancers within the first-degree relatives; B: a case with two or more colorectal cancers within the first-degree relatives meeting the following criteria: (1) age onset of colorectal cancers being earlier than 50 years old; (2) with right colon involvement; (3) with synchronous or metachronous multiple colorectal cancers; (4) associated with synchronous or metachronous extracolonic malignancies.

Germline mutations of six genes involved in DNA mismatch repair (MMR), i.e., hMSH2, hMLH1, PMS1, PMS2, MSH6 (also known as GTBP) and MLH3, have been identified in patients with the disease, and the former two genes account for the large majority of mutations found in families with HNPCC. Totally, these genes are now believed to account for about 50-70% of all families with HNPCC and over 90% of the identified mutations focused on the two genes, hMSH2 and hMLH1^[8-14]. There are many studies about the procedures of genetic testing of HNPCC, such as microsatellite instability (MSI), immunohistochemistry (IHC) and direct DNA sequencing^[15-18].

It is of no doubt that there is a large population of HNPCC in China^[19]. In 1996, Mo *et al*^[20] first reported the clinical features of HNPCC cases. Until now there have been only some case reports of HNPCC in China and no systemic study of molecular genetic aspects of HNPCC has been presented. In the present study, 16 Chinese HNPCC families are included of which nine families fulfilling the Amsterdam criteria and seven families fulfilling the Japanese criteria B. We conducted clinicopathological and molecular genetic analyses of Chinese HNPCC families.

MATERIALS AND METHODS

Materials

From 1999 to 2004, 16 Chinese HNPCC families that were registered at the Department of Surgical Oncology in the Second People's Hospital of Wenzhou and at the Department of Surgery in the First Affiliated Hospital of Wenzhou Medical College were collected, of which nine HNPCC families fulfilled the Amsterdam criteria and seven HNPCC families fulfilled the Japanese criteria B. When the probands were verified, we investigated the more detailed family history of patients in the hospital or through inquiry by telephone, mail or visit. The tree of each family pedigree was drawn. And these HNPCC patients are being followed up.

Tumor tissues of nine identified patients, peripheral white blood cells of probands and members (over the age of 18) in the five genetic analyzed kindreds were collected for the study.

Methods

Microdissection and DNA extraction The 7- μ m paraffin-embedded sections were deparaffinized. They were lightly stained with hematoxylin for microdissection. The microdissection was performed under the dissection microscope with a scalpel. The microdissected tissues were transferred directly into a centrifugation tube with 150 μ L cell lysis buffer (0.5 mol/L Tris, 20 mmol/L EDTA, 10 mmol/L NaCl, 10 g/L SDS, 0.5 g/L proteinase K).

The subsequent DNA extraction was performed according to the protocol of the DNA extraction kit (Daxia Biotech Ltd, Shanghai). So was Genomic DNA from peripheral white blood cells.

Microsatellite instability analysis Matched normal and tumor DNA were investigated with a panel of five microsatellite markers (including BAT26 and BAT25, D2S123, D5S346 and D17S250) that were recommended by the International Collaborative Group on Hereditary Non-Polyposis Cancer and the National Cancer Institute^[21,22]. The primer sequences have been published elsewhere^[22]. The primer pairs were synthesized by Shenyou Biotech Ltd. Each forward primer was labeled with a fluorescent dye at 5' end. The mixture was denatured, snap cooled and electrophoresed on ABI 310 automated DNA sequencer according to the manufacturer's instructions. The electrophoresis results were analyzed by GeneScan software. MSI was determined according to Gebert *et al*^[23]. Additional peaks (bands) at a microsatellite locus in the tumor compared with the normal tissue from the same patient were interpreted as MSI. Cases with MSI in more than two of the five loci were interpreted as exhibiting high microsatellite instability (MSI-H).

Immunostaining for hMSH2 and hMLH1 Immunostaining was performed using a monoclonal antibody against the hMSH2 (Oncogene Ltd) and a monoclonal antibody against the hMLH1 (Pharmingen Ltd) at 1:40 dilutions. The antibodies were detected by the Envision two-step method. The slides were counter stained with hematoxylin. Infiltrating lymphocytes as well as normal colonic crypt epithelium next to the tumor area served as internal positive controls. Diminished expression of hMSH2 or hMLH1 in cancer tissues were demonstrated when there was complete absence of detectable nuclear staining of neoplastic cells.

Sequencing analysis All 19 exons of hMLH1 gene and all 16 exons of hMSH2 gene (including all intron-exon borders) from proband's genomic DNA were individually amplified. PCR reaction was set in 25 μ L volume containing 100 ng template DNA, dNTPs 0.2 mmol/L, MgCl₂ 1.5 mmol/L, Taq polymerase 1 U, and then denatured for 5 min at 94 °C, cycled (45 s at 94 °C, 45 s at 55 °C and 45 s at 72 °C) 35 times and extended for 7 min at 72 °C. The PCR products were purified using the QIAquick-spin PCR purification kits (Qiagen Inc.) and then performed on an ABI 310 automated sequencer (ABS Inc.).

RESULTS

Clinicopathological characteristics

From 1999 to 2004, we investigated 16 Chinese HNPCC families of which nine families fulfilled the Amsterdam criteria and seven families fulfilled the Japanese criteria B. A total of 56 patients developed malignant tumors, 50 of whom had CRC. There were 37 male and 13 female, age ranging from 19 to 73 years (average 45.7), 36 patients (72%) developed CRC below 50 years, including 17 patients (34%) under 40 years of age, four patients (8%) under 30 years of age. 40.9% and 28.7% of the colorectal cancers' loci were located in the colon proximal to spleen flexure and in rectum, respectively. The remaining loci were not

clear. Synchronous and metachronous colorectal cancer occurred in 19 (38%) patients. We reviewed the HE of 32 cases available. From Table 1 we found 11 (34.4%) cases of poor differentiation, eight (25%) cases of mucinous adenocarcinoma. Fourteen extracolonic tumors of 13 patients were found in nine Lynch syndrome II families, including four hepatic cancers, three endometrial carcinomas, three breast cancers, two stomach cancers, one bladder cancer, and one neck cancer. Hepatic cancer is the most frequent extracolonic cancer in our series, accounting for 30.7%. Twenty-nine patients have survived for 1-28 years, 17 (58.6%) of 29 patients have survived for more than 5 years, 12 (41.4%) of 29 patients have survived for more than 10 years, 7 (24.1%) of 29 patients have survived for more than 15 years. Twenty-one patients whose median survival time was 5.7 years died during 1-23 years.

Microsatellite instability

All nine tumors in the five HNPCC families showed MSI at more than two loci (MSI-H). Four tumors showed MSI in 5/5 loci, two tumors displayed MSI in 4/5 loci, two tumors presented MSI in 3/5 loci, the other had MSI in 2/5 loci (Table 1, Figure 1).

Expression of hMSH2 and hMLH1 protein

Lack of hMLH1 immunostaining was observed in tumors from H2 proband. Tumors from probands of H1 and H7 were negative for hMSH2 immunostaining. Normal expression of hMSH2 and hMLH1 protein was observed in tumors from H5 and H6 proband (Table 1, Figure 2).

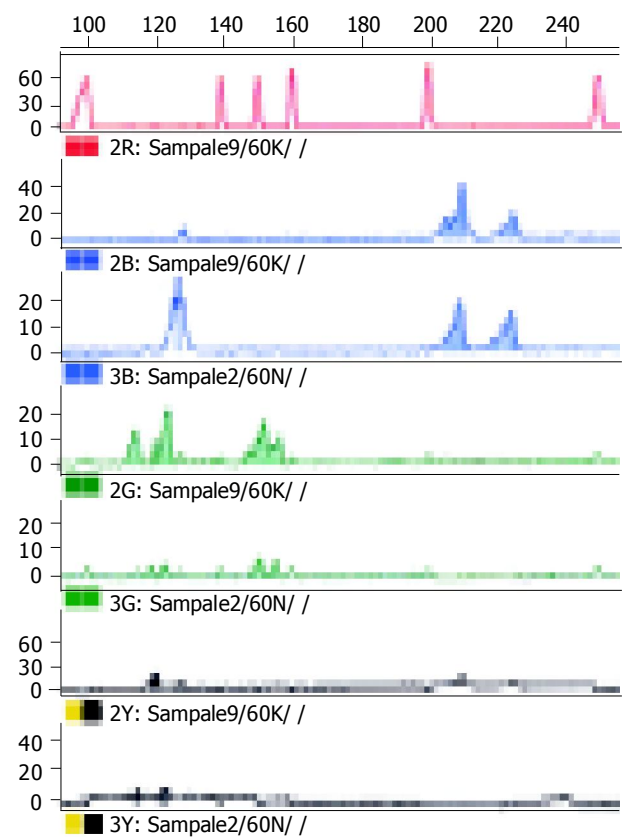


Figure 1 MSI status of H1 proband. Microsatellite analysis of H1 proband with five microsatellite markers, MSI in 5/5 loci.

Table 1 MSI status, immunostaining of nine tumors in five HNPCC kindreds

Family	Criteria	Tumor	BAT26	D2S123	BAT25	D3S346	D17S250	MSI status	Determination	hMSH2 protein	hMLH1 protein
H1	AC	CRC	+	+	+	+	+	5/5	MSI-H	Negative	N
H2	AC	CRC	+	+	+	+	+	5/5	MSI-H	N	Negative
H5-1	AC	CRC	+	+	+	+	+	5/5	MSI-H	N	N
H5-2	AC	CRC	+	-	+	+	+	4/5	MSI-H	N	N
H5-3	AC	CRC	+	NR	+	NR	NR	2/2	MSI-H	N	N
H6	JC	CRC	+	+	+	-	-	3/5	MSI-H	N	N
H7-1	AC	CRC	+	+	+	+	+	5/5	MSI-H	Negative	N
H7-2	AC	CRC	+	NR	+	+	+	4/4	MSI-H	Negative	N
H7-3	AC	CRC	-	+	+	-	+	3/5	MSI-H	Negative	N

AC: Amsterdam criteria; JC: Japanese criteria; NR: no result; MSI-H: high-level microsatellite instability; N: normal expression protein.

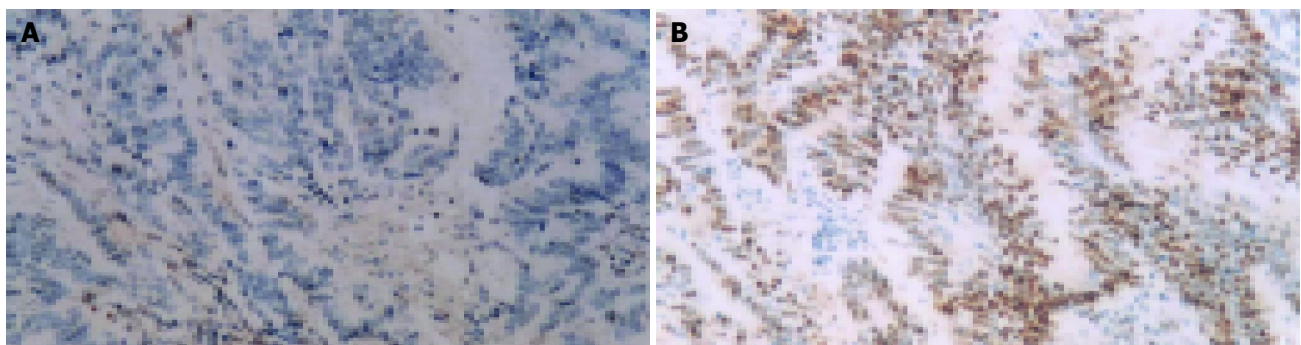


Figure 2 A: Immunohistochemical staining. Lack of hMSH2 protein expression in carcinoma area of H1 proband tumor section. $\times 400$; B: Immunohistochemical staining. Normal expression of hMLH1 protein in tumor from H5 proband. $\times 200$.

Germline mutation of hMSH2 and hMLH1 gene

Germline mutations were found in three of the five HNPCC families (60%). The first pathological mutation was a transition from C to A in exon 14 (codon 743) of hMSH2 (family H7). The second mutation was a TTC deletion in exon 14 (codon 530) of hMLH1 (family H5). The third mutation was a transition from G to A in intron 15-exon 15 borders of hMLH1 (family H6)(Figure 3). All the three were definitely pathological mutations, of which the former two had not been reported previously. All three mutations give rise to protein truncation or protein structure alteration. In addition, the affected sister and father of H7 proband also carried the same mutation in exon 14 of hMSH2. Two sons of H5 proband also suffered from colorectal cancer at young age, both carrying the same germline mutation as their father did.

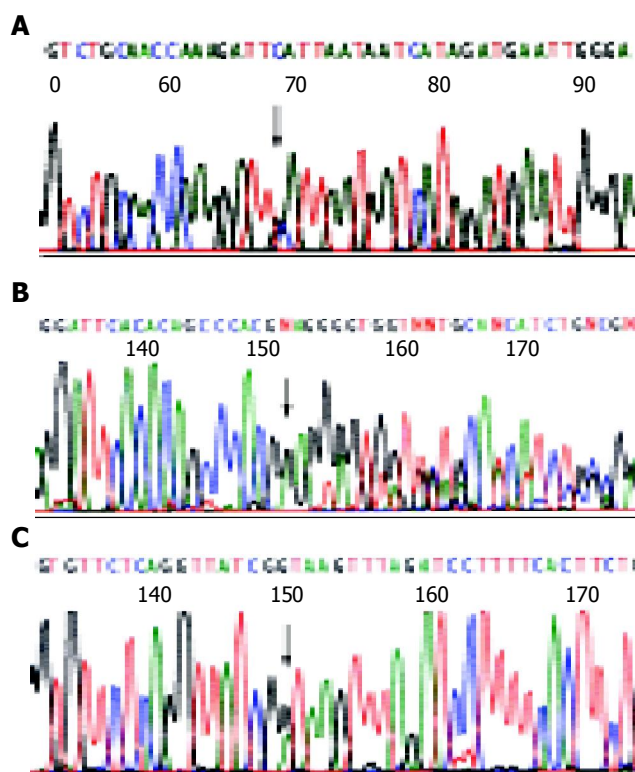


Figure 3 A: C-A transition (TCA-TAA) at codon 743 in exon 14 of hMSH2 gene in H7 proband; B: TTC deletion at codon 530 in exon 14 of hMLH1 gene in H5 proband; C: G-A transition (TCGgta → TCGata) at intron 15-exon 15 borders of hMLH1 gene in H6 proband.

DISCUSSION

HNPCC is an autosomal, dominantly inherited disease characterized by the development of cancer at an early age^[5,24-27], predominance of proximal colonic cancer^[26,28-30], excess of multiple cancers^[31-33], poorly differentiated cancer^[34], an increased risk for selected extracolonic adenocarcinomas^[35] and better prognosis^[36,37].

Early age of cancer onset is one of the most striking features about HNPCC. The average age to develop colorectal cancer was 45 years, 20 years earlier than the sporadic colorectal cancer. The study involving 43 HNPCC

kindreds and 140 HNPCC patients by Bertario *et al*^[26], showed that the average age of onset was 49 years. Cai *et al*^[19], reported the mean age of cancer onset in 30 Chinese HNPCC families, which was 44.1 years in 140 patients, with 74.6% under 50 years of age and 8.5% under 30. In the present study, the median age of onset of the first colorectal cancer was 45.7 years, with 72% under 50 years of age and 8% under 30. Moreover, we found that the age of onset of the first colorectal cancer decreased generation after generation. So to a relatively young colorectal cancer patient, especially younger than 50 years, special attention must be paid while inquiring about family history.

HNPCC is inclined to be located in the proximal colon. Colon cancers are more often right-sided, constituting 47.8% of total cancer and 74.6% of colorectal cancers^[5]. A Swedish national investigation in 2001 showed that the proportion of cancers located in the proximal colon was 51% of the total^[38]. In our study, the colorectal cancer is found proximal to the splenic flexure constituting 40.9% of the total cancer. Because of the early age of the onset of cancer and as most of the tumor would develop in the proximal colon, we recommend that colonoscopy be performed for the family members with HNPCC and repeated annually or biannually thereafter from the age of 25. In this series, we found three colon cancers and five adenomas by colonoscopy in the family members with HNPCC.

HNPCC showed tendencies of multiple synchronous and metachronous colon cancer^[27,32,33]. Fitzgibbons reported that the percentage of synchronous and metachronous colorectal cancers were 18.1% and 24.2%, respectively. It was higher than sporadic colon cancer, being 4.8% and 7.2%, respectively. And the difference was statistically evident^[5,27]. Cai *et al*, reported that 23 (19.5%) of 118 patients presented with multiple cancers in the colorectal cancer^[19]. Zhao *et al*, reported that 39.5% of colorectal cancer patients developed metachronous colorectal cancer within 10 years after their initial colorectal cancer resection. In our series, 19 of 50 (38%) patients presented with multiple cancers in the colorectal cancer. The high incidence of multiple cancers implies that subtotal colectomy is an appropriate management when colon cancers are found in affected patients. It can reduce the chance of developing synchronous colorectal cancer and simplify the endoscopic examination^[27]. But considering the effect on the quality of life after subtotal colectomy and the psychological attack on the patients, we usually chose segmental resection for colorectal carcinoma and gave intensive follow-up.

Extracolonic tumors were often seen in HNPCC kindred^[27,35,39,40], such as carcinomas of endometrium, ovary, stomach, small bowel, urologic system, hepatobiliary system, breast, brain, larynx, pancreas and as well as leukemia, lymphoma, soft tissue sarcoma, and cutaneous tumors. According to the reports of Western countries, the endometrial and stomach cancers are the first and the second most common tumor in HNPCC, respectively^[33,41,42]. In our series, 14 extracolonic tumors of the 13 patients in the nine Lynch syndrome II families were found, of which four were hepatic cancers, three endometrial carcinomas, three breast cancers, two stomach cancers, one bladder cancer,

one neck cancer. Hepatic cancer is the most frequent extracolonic cancer in our series, accounting for 30.7%. Zhao *et al.*, in China reported 34 cases of extracolonic cancer in 16 HNPCC families. He also found that stomach cancer was the most common extracolonic tumor in HNPCC (11 cases of stomach cancers) and endometrial cancer was less common (seven of 34) than gastric cancer. The difference in extracolonic tumor spectrum between China and Western countries may lie in many aspects. The small size of Chinese sample may be one of the reasons. Besides, life style, ethnicity and genotype may also contribute to the observed variation.

They were recognized by histopathological criteria in HNPCC: (1) mucinous histotype; (2) poorly differentiated tumors; (3) presence of peritumoral lymphocytic infiltrate, with Crohn's-like lymphoid reaction. Jass *et al.*^[34], reported that there is an excess of poorly differentiated and mucinous tumors in HNPCC. Similarly, in our series, poor differentiation cancers and mucinous adenocarcinoma accounted for 34.4% and 25%, respectively. But, there are rarely mucinous histotypic presence of peritumoral lymphocytic infiltrate, with Crohn's-like lymphoid reaction in our study, perhaps it was due to the small sample of our study or the Chinese racial difference.

Patients with HNPCC have been suggested to have a better prognosis than patients with common sporadic colorectal cancer. Sankila *et al.*, compared the survival rates of 175 patients with HNPCC with those of 14000 patients with sporadic colorectal cancer diagnosed at <65 years of age in Finland from 1953 to 1993. They showed that the overall five-year cumulative relative survival rate was 65% for patients with HNPCC and 44% for patients with sporadic colorectal cancer^[36]. In our study, 21 patients whose median survival time was 5.7 years died during 1-23 years. Twenty-nine patients have survived for 1-28 years, 58.6%, 41.4% and 24.1% patients have survived for more than 5, 10 and 15 years, respectively. The better survival rates may be caused by the heavy mutation burden affecting MMR-deficient tumor cells^[36]. Takemoto *et al.*^[37], suggest that there might be a possibility of ITCIL having a role for a better prognosis after colorectal cancer surgery, which is closely related to MSI. But there is a conflicting data existing on the prognosis of hereditary colorectal cancer. Bertario *et al.*^[26], found no substantial survival advantage for HNPCC patients compared with the sporadic group, after adjustment for age, gender, stage and tumor location.

Microsatellite has been widely considered as an ideal genetic marker. MSI reveals loss of the function of MMR genes. It can serve as a reliable preliminary screening strategy of HNPCC family as several studies have shown that MSI occurs in about 80-90% of HNPCC tumors^[43-47]. In the current study, we adopted a panel of five sensitive microsatellite markers accepted by the International Collaborative Group for HNPCC and the National Cancer Institute to detect the MSI status^[48]. One hundred percent (9/9) of the nine tumors displayed high-level MSI, which showed high-level defection of MMR function in the affected patients from HNPCC families fulfilling Amsterdam or Japanese criteria B. Sixty percent of the families that have affected patients with high-level MSI were found with germline mutation of

hMLH1 or hMSH2 gene, which showed that MSI status and germline mutation of hMLH1 and hMSH2 gene correlated closely with each other.

Ninety percent of HNPCC cases are associated with the mutation of hMSH2 and hMLH1 genes^[49]. Recent studies showed that the immunostaining of proteins produced by these two genes could serve as a convenient, rapid and cheap approach in screening HNPCC families^[50-52]. In our study, three tumors showed the lost expression of hMSH2 protein and a germline mutation of hMSH2 gene was identified, four tumors showed hMLH1 protein expression and a germline mutation of hMLH1 gene was identified. Two tumors showed that no pathological germline mutation had been detected, but one tumor displaying no expression of hMSH2 protein, one tumor showed the lost expression of hMLH1 protein. In general, immunohistochemical alteration of hMSH2 and hMLH1 proteins and germline mutation of hMLH1 and hMSH2 gene correlated closely with each other. IHC is also very useful in screening HNPCC families.

Direct gene sequencing remains the most reliable method for HNPCC diagnosis. Till now about 400 different predisposing mutations have been reported, mainly affecting the MMR genes hMLH1 (about half), hMSH2 (about 40%) and MSH6 (about 10%)^[49]. There appeared no hot spot mutations among those found in these mutations. The three mutations (one in hMSH2 and two in hMLH1) found in five collective families are all pathological mutations. The rate of mutation is 60% (3/5). The first pathological mutation in H7 proband was a transition from C to A in exon 14 (codon 743) of hMSH2. The second mutation in H5 proband was a TTC deletion in exon 14 (codon 530) of hMLH1. The third mutation in H6 proband was a transition from G to A in intron 15-exon 15 borders of hMLH1. The former two pathological mutations had not been reported before (<http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html>). All three mutations gave rise to protein truncation or protein structure alteration. The mutations existed also in genomic DNA from other affected family members. For example, the affected sister of H5 proband also carried the same mutation in exon 14 of hMSH2. Two sons of H7 proband also suffered from colorectal cancer at the age of 37 and 38, both carrying the same germline mutation as their proband did, the other son was not found to have any tumor till the age of 43 and also his daughter till the age of 46, both the same germline mutation had not been identified. All new mutations appearing in our study demonstrates the wide spectrum of the mutation responsible for HNPCC. MMR gene mutation analysis will give both HNPCC proband and his family members better management and surveillance, and it will also support genetic counseling as well as gene therapy in the future. For the proband himself, it is helpful for us to conduct positive and effective therapy to reduce the occurrence of possible metachronous multiple colorectal cancer. To the mutation carriers in a family who have not yet suffered from colorectal cancer, close follow-up and early diagnosis are more likely to be performed. To the non-mutation carriers, we should free them from unnecessary psychological and economical burden^[50]. But the mutation may be different in a variety

of races and geographical regions. Mutations of hMSH2 and hMLH1 accounted for 25-86% of the total cases^[40,53,54]. A deeper study of germline mutation remains a heavy assignment for us.

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