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BRIEF ARTICLES

Genetic diagnosis strategy of hereditary non-polyposis colorectal cancer

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Abstract

AIM: To study the characteristics of mismatch repair gene mutation of Chinese hereditary non-polyposis colorectal cancer (HNPCC) and hMLH1 gene promoter methylation, and to improve the screening strategy and explore the pertinent test methods.

METHODS: A systematic analysis of 30 probands from HNPCC families in the north of China was performed by immunohistochemistry, microsatellite instability (MSI), gene mutation and methylation detection.

RESULTS: High frequency microsatellite instability occurred in 25 probands (83.3%) of HNPCC family. Loss of hMLH1 and hMSH2 protein expression accounted for 88% of all microsatellite instability. Pathogenic mutation occurred in 14 samples and 3 novel mutational sites were discovered. Deletion of exons 1-6, 1-7 and 8 of hMSH2 was detected in 3 samples and no large fragment deletion was found in hMLH1. Of the 30 probands, hMLH1 gene promoter methylation occurred in 3 probands. The rate of gene micromutation detection combined with large fragment deletion detection was 46.7%-56.7%. The rate of the two methods in combination with methylation detection was 63.3%.

CONCLUSION: Scientific and rational detection strategy can improve the detection rate of HNPCC. Based on traditional molecular genetics and combined with epigenetics, multiple detection methods can accurately diagnose HNPCC.

Key words: Hereditary non-polyposis colorectal cancer; Gene mutation; Mismatch repair; hMSH2; hMLH1; Large fragment deletion; Methylation

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INTRODUCTION

Hereditary non-polyposis colorectal cancer (HNPCC) is a dominant autosomal genetic syndrome caused by germ-line mutation of mismatch repair gene[1], accounting for 5%-10% of all colorectal cancer[2,3]. Genetic linkage analysis and genetics show that about 80% of HNPCC are associated with germ-line mutation of hMLH1 and hMSH2[4-6]. Most of these mutations are micromutation (also known as a point mutation), small fragment insertion or deletion, *etc*[7,8]. Large fragment deletion in hMLH1 and hMSH2 (especially hMSH2) gene is another way of germ-line mutation[9]. In addi-

tion, recent epigenetic studies indicate that CpG island methylation in hMLH1 gene promoter region is also a mechanism underlying gene inactivation and tumorigenesis[10]. Therefore, systematic and comprehensive detection analysis of 30 samples from HNPCC families in the north of China was performed using various methods.

MATERIALS AND METHODS

Materials

Between 2000 and 2002, 30 probands from HNPCC families were collected and registered by General Hospital of Beijing Military Command and other hospital in Henan, Hebei and Shandong Provinces, and Inner Mongolia Autonomous Region. All study methods were approved by the Ethics Committee of General Hospital of Beijing Military Commad and the 30 probands from HNPCC families gave written consent to participate in the study. Twenty-one families were in line with Bethesda Guideline (BG) Ⅰ, 7 families Bethesda Guideline Ⅲ and 2 families Bethesda Guideline Ⅳ, respectively[11]. With probands as the core, HNPCC genealogical tree of at least two generations was drawn. Pedigree analysis was performed. Family member files including the age when the tumor was diagnosed, the relation to proband, tumor type and location, *etc* (including parenteral cancer) were established.

Methods

DNA extraction: Tumor and normal tissues were obtained from probands. Tumor tissue was fixed with formalin, embedded in paraffin, cut into 6-μm thick sections which were stained with 0.1% methylthioninium chloride. The location (more than 80% tumor tissue) most suitable to microdissection was marked and microdissection was done. DNA was extracted from tumor and normal tissues with phenol/chloroform/isoamyl alcohol for microsatellite instability (MSI) and immunohistochemistry detection[12]. DNA was extracted from venous blood for gene mutation and methylation detection.

Microsatellite instability and immunohistochemistry detection: Microsatellite instability detection was performed in 5 microsatellite markers: D2S123, D5S346, BAT-25, BAT-26 and BAT-40[13,14]. For hMSH2 and hMLH1, immunohistochemical staining was done with the standard biotin-avidin-peroxidase complex method as previously described[15].

hMSH2 and hMLH1 gene mutation detection: Micromutation detection was performed in 25 samples with high microsatellite instability. PCR was performed to amplify all exons (including intron-exon junction) of hMSH2 and hMLH1. PCR products were sequenced with a DNA automatic sequencer (ABIPRISM 3730XL) to find micromutation in the samples and to determine their mutation type.

Large fragment deletion detection in hMSH2 and hMLH1: Multiplex ligation-dependent probe amplification (MLPA) technique[16] was used to detect large fragment deletion in the samples without micromutation with a hMLH1 and hMSH2 large fragment deletion kit purchased from MRC Holland Company. The major steps of MLPA technique include to probe hybridization with the target sequence and specific ligation, to amplify hMSH2 and hMLH1 by PCR with probes, and to analyze PCR products. The PCR products were applied to a ABIPRISM3730 sequencer containing 6% polyacrylamide gel for electrophoresis. The electrophoresis results were analyzed with GeneMapper 3.0 software. The peak of each exon was compared with that of control sample. If the relative height was reduced by 35%-55%, the fragment was determined to have exon deletion. If the relative height was increased by 30%-55%, the fragment was determined to have exon duplication. If the peak was 0, the fragment was determined to have homozygous deletion.

Methylation detection: hMLH1 gene promoter methylation detection was performed in 30 samples. First, DNA was sulfurized with an EZ DNA methylation-gold kit purchased from ZYMO RESEARCH Company, and then methylation-specific PCR (MSP)[17] was performed. PCR amplification was performed twice for each sample with methylation and non-methylation primers, respectively. PCR products were applied to a 10% non-denaturing polyacrylamide gel for electrophoresis, then stained with ethidium bromide, and observed under an ultraviolet lamp.

RESULTS

Clinical and pathological information and family history

Of the 30 families, 21 were in line with BG Ⅰ, 7 were in line with BG Ⅲ and 2 were in line BG Ⅳ (Table 1). One hundred and forty tumors were found in 106 of the 708 members in these families. Of the 140 tumors, 22 (15.7%, 22/140) were extracolonic cancers. Of the 22 tumors, 7 were gastric cancers which are the most common type of extracolonic cancer. Of the colon cancers, 86 (72.9%, 86/118) were right colon cancers and 32 (27.1%, 32/118) were left colon cancer. One patient had synchronous multiple-primary cancers and 7 had metachronous multiple-primary cancers.

Microsatellite instability analysis and mismatch repair protein expression

Microsatellite instability analysis and mismatch repair protein expression in probands are shown in Table 1. Of the 30 samples detected, high frequency microsatellite instability (MSI-H) occurred in 25 samples (83.3%), low frequency microsatellite instability (MSI-L) in one sample (3.3%) and microsatellite stability (MSS) in 4 samples (13.3%). Of the 5 microsatellite loci, MSI-H expression rate was 100% (25/25) and 96% (24/25) in BAT-25 and BAT-26, respectively. Of the 25 samples with MSI-H, loss of hMLH1 or hMSH2 protein expression occurred in 22 (88%), loss of hMLH1 protein expression occurred in 12, and loss of hMSH2 protein expression in

Table 1 Detection results in 30 probands from HNPCC families

Family	Bethesda guidelines	MSI	Expression of MMR proteins		Micromutation/polymorphism[2]/large fragment deletion		hMLH1 gene promoter methylation
			MSH2	MLH1			
H1	BG1	MSI-H	+	+			u
H4	BG1	MSI-H	-	+	hMSH2 exon13	IVS13-2 A→C (SA of Exon 14)	u
H9	BG1	MSI-H	-	+	hMSH2 exon3	c.610G→T (G204stop)	u
H17	BG1	MSI-H	+	+	hMSH2 exon5	c.899_890insAT1	u
H22	BG1	MSI-H	-	+	hMSH2 exon7	IVS7-1G→A (SA of Exon 8)[1]	u
H10	BG1	MSI-H	+	+	hMSH2 exon15	c.2583A→G (Q861Q)[1,2]	u
H2	BG1	MSI-H	-	+	hMSH2 exon8	deletion	u
H5	BG1	MSI-H	-	+			u
H11	BG3	MSI-H	-	+			u
H23	BG1	MSI-H	-	+	hMSH2 exon1-6	deletion	u
H25	BG3	MSI-H	-	+			u
H13	BG1	MSI-H	-	+	hMSH2 exon 7	c.1231 insertion T shift	u
H34	BG4	MSI-H	-	+	hMSH2 exon1-7	deletion	u
H3	BG1	MSI-H	+	-	hMLH1 exon18	c.2041G→A (A681T)	u
H12	BG3	MSI-H	+	-	hMLH1 exon15	IVS15+1 G→A (SD of Exon 15)	u
H19	BG1	MSI-H	+	-	hMLH1 exon8	c.677G→A (splice site mutation)	u
H20	BG1	MSI-H	+	-	hMLH1 exon8	c.677G→A (splice site mutation)	u
H21	BG1	MSI-H	+	-	hMLH1 exon19	c.2141G→A (W714stop)	u
H28	BG1	MSI-H	+	-	hMLH1 exon8	c.655A→G (I219V)	m
H29	BG1	MSI-H	+	-	hMLH1 exon6	c.503_4insA1	u
H30	BG1	MSI-H	+	-	hMLH1 exon9	IVS9+1 G→A (SD of Exon 9)	u
H14	BG1	MSI-H	+	-			u
H18	BG1	MSI-H	+	-			u
H33	BG3	MSI-H	+	-			u
H35	BG3	MSI-H	+	-	hMLH1 exon17	c.1930 del G	u
H8	BG4	MSI-L	+	+			m
H6	BG3	MSS	+	+			m
H36	BG1	MSS	+	+			u
H15	BG3	MSS	+	+			u
H27	BG1	MSS	+	+			u

+: Expression; -: Deletion; [1]Novel mutation discovered; [2]Polymorphism; m: Methylation; u: Non-methylation; MSI-H: High frequency microsatellite instability; MSI-L: Low frequency microsatellite instability; MSS: Mcrosatellite stability.

10, respectively. No loss of mismatch repair protein expression was found in the other 8 samples.

DNA sequencing

Of the 25 samples with MSI-H, pathogenic mutation (Table 1) was detected in 14 (56%). Of the 14 samples, hMLH1 and hMSH2 gene mutation occurred in 9 and 5, respectively. The detection rate of micromutation was 46.7% (14/30). Three novel mutational sites were discovered. Of the 3 novel mutations, a frame shift mutation at c.503_4insA was located in hMLH1, and another frame shift mutation at c.899_890ins AT and a splicing mutation at IVS7-1G→A, SA of Exon 8 were located in hMSH2. A new base replacement (hMSH2, c.2583A →G) was detected in sample H10, which did not cause changes in amino acid sequence. There was no co-segregation phenomenon between this base replacement and disease in its family, suggesting that it is a new change in polymorphism.

Large fragment deletion detection

Electrophoresis scanning peaks in exons 1-6, 1-7 and 8 of hMSH2 in 3 samples were reduced by over 35% (Figure 1), demonstrating that these exons have deletions which are heterozygotic in nature. Large fragment deletion was not detected in hMLH1. Large fragment deletion in hMSH2 accounted for 37.5% of all hNSH2 pathogenic mutations and 17.6% of total hMLH1 and hMSH2 mutations, respectively. Of the 30 samples, micromutation and large fragment deletion were detected in 17, the detection rate of combined methods was 56.7% (17/30).

Methylation detection

hMLH1 gene promoter methylation occurred in 3 of the 30 samples. The detection rate of combined micromutation, large fragment deletion and methylation detection was 63.3% (19/30). MSP electropherogram (Figure 2) showed that hMLH1 gene promoter methylation occurred in 3 samples. Exhaustive methylation occurred in 2 of the 3 samples with their electropherograms displaying M specific fragment but no U deletion, and partial methylation occurred in one of the 3 samples with its electropherogram displaying M and U fragments. Only U specific fragment occurred in the other samples, indicating that no methylation occurs in these samples.

DISCUSSION

Germ-line mutation of mismatch repair gene is the molecular genetic basis of HNPCC pathogenesis. Mismatch repair gene mutation may lead to truncation

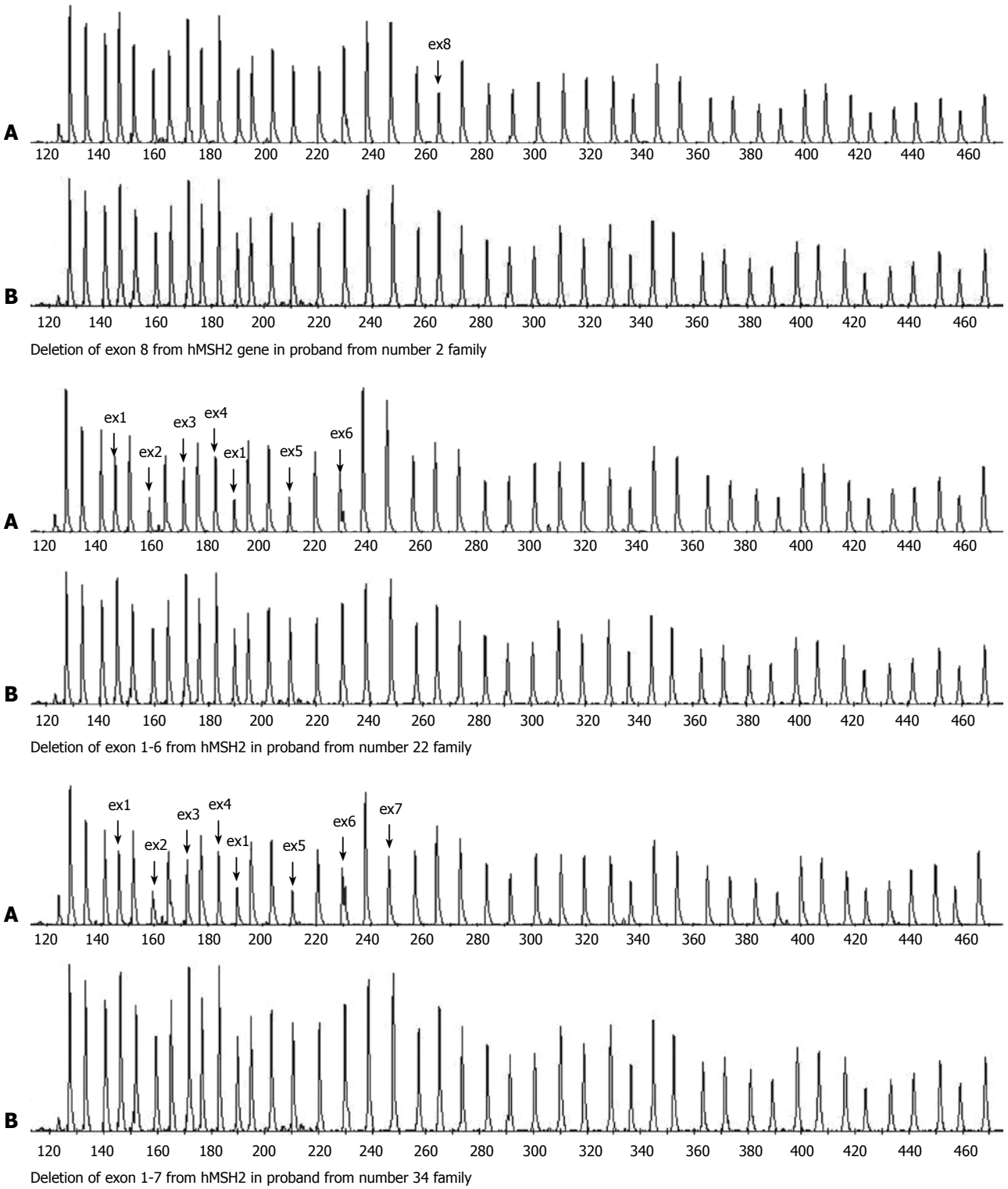

Figure 1 Large fragment deletion results with MLPA technique. A indicates peak graph of fluorescence intensity of proband PCR products. B indicates peak graph of fluorescence intensity of control PCR products. ↓ indicates large fragment deletion in exon.

and lower expression of mismatch repair protein, increasing DNA replication errors and microsatellite instability which results in tumorigenesis[18,19]. Mutations in hMSH2 and hMLH1 are most common[4-6]. Therefore, we mainly studied the two genes.

Microsatellite instability detection was performed in 5 microsatellite markers, including D2S123, D5S346, BAT-25, BAT-26 and BAT-40. The MSI-H detection rate was 83.3%, demonstrating that the incidence of MSI-H is high in patients with HNPCC, and microsatellite instability is one of the most important features of HNPCC and reflects mismatch repair gene state at a certain extent. Loss of mismatch repair protein expression occurred in 88% MSI-H samples while no loss of mismatch repair protein expression occurred in MSI-L and MSS samples, indicating that the specificity, sensitiv-

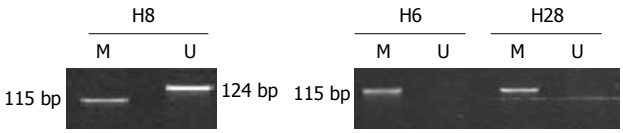

Figure 2 Electropherogram of hMLH1 gene promoter methylation detection. M: Methylation; U: Non-methylation; H: Family number. H8 manifests partial methylation. H6 and H28 manifest exhaustive methylation.

ity and consistency of MSI and immunohistochemistry detection are higher in HNPCC families, the detection methods are simple and economic, and the results of combined detection methods may be used as effective screening indicators before genetic testing.

In MSI-H samples, the detection rate of micromutation in hMSH2 and hMLH1 was 56%. Micromutations of hMLH1 occurred in exons 6, 8, 9, 15, 17-19 and accounted for 64.3%. Micromutations of hMSH2 occurred in exons 3, 5, 7 and 13 and accounted for 35.7%. The mutation types included frame shift, non-sense, splicing and missense mutations. By searching the database of International Society for Gastrointestinal Hereditary Tumors (http://www.insight-group.org), we found 3 new mutational sites including a frame shift mutation at c.503_4insA in hMLH1, and a frame shift mutation at c.899_890ins AT, and a splicing mutation at IVS7-1G→A, SA of exon 8 in hMSH2. Since these mutations could lead to protein product truncation, further study is needed in a larger-scale population to confirm whether such mutations only occur in the north of China. It is evident that the mutation spectrum of HNPCC mismatch repair gene in Chinese is broad and multiple.

Grabowski *et al*[20] reported that large fragment deletion in hMLH1 and hMSH2 accounts for 17% of all pathogenic mutations, which is almost consistent with the results of our study. The three types of large fragment deletion are in line with the findings reported by Nakagawa *et al*[21]. Large fragment deletion commonly occurs in hMSH2, and should be reckoned with in molecular genetics of HNPCC. Therefore, detection of HNPCC molecular genetics should include large fragment deletion detection.

With the development of epigenetics in recent years, DNA methylation has gradually become a new research focus. In human genome, 5' promoter of 50% genes contains a CpG region, also known as CpG island, with its length > 197 bp. CpG island is in a non-methylation state under normal circumstances. CpG island methylation may lead to loss of gene expression and replication errors[22,23]. The promoter methylation of hMLH1 gene is most common in known mismatch repair genes. The detection rate of hMLH1 gene promoter methylation in our study was almost similar to the reported data[24]. Exhaustive and partial methylations were observed in our study, demonstrating that hMLH1 gene promoter methylation may occur in patients with HNPCC, and the methylation level is different in different individuals. A deletion at c.655A→G (1219 V) in hMLH1, loss of hMLH1 protein expression, and hMLH1 gene promoter methylation were found in sample H28, suggesting that further study is needed to explore the protein expression and regulation of HNPCC pathologic mechanism.

An overview of the whole process of detection analysis of the 30 proband samples, microsatellite instability was first performed, then gene micromutation, large fragment deletion and promoter methylation were detected, respectively. The detection rate of gene micromutation was 46.7% (14/30), the detection rate of large fragment deletion detection in combination was increased to 56.7% (17/30). However, the detection rate of three methods in combination was 63.3% (19/30). Almost no comprehensive and systemic detection has been reported both at home and abroad. The detection rate in our study was higher than or similar to reported data[25-27]. Multiple methods in combination may improve the detection efficiency and accuracy of HNPCC and can determine HNPCC families. In order to make early diagnosis and treatment, HNPCC family members should regularly by examined. At the same time, since gene detection is time-consuming and expensive, the cost of various tests and clinical significance should be taken into account according to the actual situation. Therefore, the detection strategy should be made in the following steps. First, families meeting the HNPCC criteria are selected, and then immunohistochemistry and microsatellite instability detection of hMLH1 and hMSH2 are performed. If both of the two detections are negative, mutation detection need not be done. If one of the two detections is positive, micromutations in hMLH1 and hMSH2 should be detected. If micromutation is not detectable, large fragment deletion detection should be considered. MLPA technique can be used in detecting large fragment deletion and is characterized by DNA probe hybridization. PCR technique is rapid, sensitive, specific, reliable and cheap. All the 35 exons of hMLH1 and hMSH2 gene can be detected in the same reaction system with MLPA technique[28]. Epigenetics provides a new idea for the early diagnosis, treatment and prognosis of tumors. Studies indicate that CpG island methylation in the hMLH1 gene promoter region is also a mechanism underlying gene inactivation and tumorigenesis[29,30]. Therefore, based on traditional micromutation detection, we should further combine large fragment deletion detection. For samples without micromutation and large fragment deletion, mismatch repair gene promoter methylation detection should be taken into account. Various detection methods in combination can better diagnose HNPCC.

COMMENTS

Background

Hereditary non-polyposis colorectal cancer (HNPCC), which is caused by a germline mutation in the mismatch repair gene or is associated with tumors exhibiting microsatellite instability (MSI), is characterized by increased risk of developing colon cancer and other cancers, such as cancers of endometrium, ovary, stomach, small intestine, hepatobiliary tract, upper urinary tract, brain, and skin. The diagnosis of HNPCC can be made based on the Amsterdam Clinical Criteria or on molecular genetic testing for germline mutations in one of the mismatch repair (MMR) genes.

Research frontiers

About 80%of HNPCC are associated with germline mutations in hMLH1 and hMSH2. Most of these mutations are micromutation that is a point mutation, small fragment insertion or deletion, *etc*. Large fragment deletion in hMLH1 and hMSH2 gene is another way of germline mutation. In addition, recent epigenetic researches indicate that CpG island methylation in the hMLH1 gene promoter region is also a mechanism underlying gene inactivation and tumorigenesis.

Innovations and breakthroughs

They used combined MSI and immunohistochemistry detection, gene mutation detection (i.e. sequencing and large fragment deletion detection) and methylation detection to study the characteristics of MMR gene mutation and hMLH1 gene promoter methylation in Chinese HNPCC patients. The results demonstrate that the combined MSI and immunohistochemistry detection might be used as effective screening indicators before genetic testing. Furthermore, detection of HNPCC molecular genetics should include large fragment deletion detection. For samples without micromutation and large fragment deletion, mismatch repair gene promoter methylation detection should be taken into account.

Applications

According to their results, the detection strategy should be made in the following steps. First, families meeting the HNPCC criteria are selected, and then immunohistochemistry and microsatellite instability detection of hMLH1 and hMSH2 are performed. If both of the two detections are negative, mutation detection needs not be done. If one of the two detections is positive, micromutations in hMLH1 and hMSH2 should be detected. If micromutation is not detectable, large fragment deletion detection should be considered. For samples without micromutation and large fragment deletion, mismatch repair gene promoter methylation detection should be taken into consideration. Various detection methods in combination may better diagnose HNPCC.

Peer review

The authors performed a systematic analysis of various genetic diagnostic methods in Chinese HNPCC patients and assessed their efficiency. The results are interesting and suggest that MSI, immunohistochemistry detection, gene mutation detection and methylation detection in combination may better diagnose HNPCC.

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