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Detection of Occult High Graded Microsatellite Instabilities in MMR Gene Mutation Negative HNPCC Tumors by Addition of Complementary Marker Analysis

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Abstract

Background: Hereditary non-polyposis colorectal cancer (HNPCC) is an autosomal dominant tumor syndrome predisposing to predominantly colorectal and endometrial cancer. In 90% of the cases, molecular analyses reveal microsatellite instabilities due to germline mutations in DNA mismatch repair (MMR) genes, mainly MLH1, MSH2, among these tumors.

Patients and Methods: Tumors from 40 HNPCC index patients (31 Amsterdam positive, 9 Bethesda positive; 21 females, 19 males; mean age 48.0 ± 13.2 years) were examined. In contrast to the classical constellation, their tumors revealed only a microsatellite stable (MSS, n = 31) - or low instable (MSI-L, n = 9) - tumor phenotype following the international reference panel of 5 microsatellites. No MLH1 and MSH2 mutations were detectable. Complementary microsatellites (BAT40, D10S197, D13S153, D18S58, MYCL1) were investigated by PCR and fragment analysis to find other instabilities which might hint to the MIN-pathway of the tumors.

Results: Due to ten microsatellites in total tumors were now reclassified in 4 MSI-H (10%), 24 MSI-L (60%) and 12 in MSS (30%) phenotypes. The mean age of onset for CRCs was the lowest in the MSI-H group with 45.7 ± 9.6 years (vs. 48.7 ± 14.3 and 49.0 ± 12.9 years in MSI-L and MSS group). MSI-H-and MSI-L tumors were often localized in the proximal colon (50 and 52%), whereas MSS tumors were preferentially localized in the distal colon (77%).

Conclusion: Complementary microsatellites help to subdive "non-classical" HNPCC in subgroups with different clinical appearance. It allows to detect occult MSI-H tumors with up to 10% and to confirm MSS tumors who seem to have a similar biological behaviour like sporadic CRC. Maybe that this genetic reclassification influence the decision of whether to offer patients chemotherapy or not, since it is known that patients with instable tumors do not benefit from chemotherapy as well as patients with microsatellite stable tumors.

Key words: HNPCC, microsatellite instability, mismatch repair genes

Introduction

Hereditary non-polyposis colorectal cancer (HNPCC), known as an autosomal dominant tumor syndrome characterized by early onset of colorectal, particularly right-sided, cancer, occurs with an incidence of up to 5% of all colorectal carcinomas (CRC) [1]. Most of these tumors show a typical genetic instability in simple genomic sequences, so-called microsatellites, due to germline mutations within the DNA mismatch repair (MMR) genes, mainly MSH2, MLH1, and MSH6 [2-5].

These MMR gene mutations preferentially occur in cases of microsatellite instability with at least two out of five markers (40%, so called MSI-high phenotype, MSI-H), whereas they have rarely been found in individuals with only one or none of these five markers, defined as MSI-low (MSI-L) or microsatellite stable (MSS) phenotype [6, 7]. The Amsterdam criteria (AC) as clinical identification features are known to have the highest predictive value for the detection of these genetic alterations [8].

The aim of our study was to investigate tumors of patients fulfilling the clinical criteria for a HNPCC syndrome (mostly Amsterdam positive) in whom the established microsatellite and mutation as well as immunhistochemical analyses did not reveal the genetic alterations.

Patients and Methods

Patients

Among 249 patients who are documented in our genetic database with a family history of HNPCC we selected 40 patients (31 patients fulfilling the Amsterdam and 9 fulfilling the Bethesda criteria) [6, 9]. In routine testing, their corresponding tumors were analyzed with five markers of the international reference panel (BAT25, BAT26, D5S346, D2S123, D17S250) and were judged to be only MSS or MSI-L phenotype. Immunohistochemistry and mutation analysis for MLH1 and MSH2 did not reveal a loss of expression of the corresponding protein nor a disease causing mutation in these tumors.

Tumors

Tumors were judged macroscopically and microscopically including histologic examination (hematoxylin and eosin staining). They were staged following the TNM classification and were graded in regard to their differentiation (G1-4).

DNA Extraction

DNA was extracted from tumor blocks by proteinase K digestion followed by repeated ethanol precipitation with gradual declining ethanol concentrations [10] using the QIAmp Tissue Kit (Quiagen, Hilden, Germany). From the same patients DNA was isolated out of peripheral blood leucocytes by using standard methods. Purified DNA was then quantified spectrophotometrically before polymerase chain reaction (PCR).

Polymerase Chain Reaction

Five complementary microsatellite markers (BAT40, D10S197, D13S153, D18S58 and MYCL1) were chosen as described [11, 12]. One member of each primer pair was labeled with a fluorescent dye to permit detection by using an automated fluorescent DNA fragmenting apparatus.

Polymerase chain reaction was performed in a final volume of 20 ml containing 100 ng DNA, 10 x PCR buffer, 20 μM of each dNTP, 3 mM Mg^{2+}, 30 pmol of each primer and 1 U of Taq DNA polymerase (Master Mix, Quiagen, Hilden, Germany). The DNA was amplified in a thermocycler (Biometra, Göttingen, Germany) using a hot-start approach. PCR initially denatured at 94 °C for 30 seconds, annealed at 55 °C for 25 seconds for BAT40 and MYCL1, at 58°C for 25 seconds for D10S197, D13S153 and D18S58, with extension at 72°C for 1 minute. The final extension after 35 cycles was performed at 72 °C for 5 minutes and was followed by cooling to 4 °C. Amplification products were visualized by ethidium bromide staining in a 1.5% agarose gel electrophoresis.

Fragment Analysis

PCR products were analyzed by a polyacrylamide/formamide gel electrophoresis in a sequencing gel chamber (ABI PRISM 377 Genetic Analyzer). Analysis was performed by a special software (PE Applied Biosystems) and included determination of the length of the PCR products and the height of the peaks. Amplification products ranged between 80 - 230 base pairs.

MSI was defined by the presence of novel bands or a band shift following PCR amplification of tumor DNA, which were not present in PCR products of the corresponding normal DNA.

Interpretation of MSI-phenotypes

A tumor was considered as having an MSI-H phenotype if at least four out of ten markers (40%) exhibited band size shifts. Samples were qualified as MSI-L phenotype if only up to three markers demonstrated genetic alteration. Tumors without band shifts were reclassified as microsatellite stable (MSS).

Mutation Analysis for MSH2 and MLH1

All exons of the MLH1 and MSH2 genes were amplified using primers published previously [13, 14] with a touch down PCR-program: 94 °C for 5 min and first annealing at 63 °C for 30 sec, then a progressive decrease of the annealing temperature by 1°C each cycle until the lowest annealing temperature of 50°C, followed by synthesis at 72 °C for 30 sec and then denaturation at 94 °C for 30 sec with another 15 cycles at an annealing temperature of 50°C.

DHPLC analysis was carried out on an automated DHPLC device equipped with a DNA separation column (WAVE: Transgenomic, San Jose, California, USA). Four to seven ml of each PCR product (containing 50-100 ng of DNA) was denatured at 95°C for 3 min. The DNA strands were gradually reannealed by decreasing the sample temperature from 95 °C to 65 °C over a period of 30 min. PCR products were then separated through a linear acetonitrile gradient (flow rate 0.9 ml/min). The column mobile phase consisted of a mixture of 0.1 M triethylamine acetate (pH 7.0) with (buffer B) or without (buffer A) 25% acetonitrile. Gradient parameters were determined based on the size and G-C content of the amplicon. Generally, analysis took approximately 7 min, including column regeneration and re-equilibration to starting conditions. The temperature for the successful resolution of heteroduplex molecules was determined by running fragment -specific melting curves and by using the DHPLC melting algorithm WAVE-MAKER of the wave instrument. Melting curves were determined as follows: the elution time of a specific fragment was determined under standard conditions. This specific gradient was then tested with the same PCR product and temperatures ranging from 48 °C to 70°C, and the retention time versus temperature was plotted to yield a fragment-specific melting curve. These combined

results revealed an analysis temperature for each melting domain of the fragment which is optimal for 80-90% of the a-helical fraction of each domain.

Immunohistochemistry for MSH2 and MLH1.

MSH2: Monoclonal mouse anti-human MSH2-antibody (Calbiochem Oncogene Research Products, Heidelberg, Germany) was used at a solution of 1 : 200. 2-μm sections of the paraffin-embedded, formalin-fixed tissue blocks were pretreated by microwaving with target unmasking fluid (TUF, DAKO, Hamburg, Germany) for 15 min at high power. The tissue was then incubated with the primary antibody overnight at 4 °C. Staining was visualized using avidin-biotin complex (Vectastain Elite ABC-Kit, Vektor Labs, Wertheim-Buettingen, Germany) with 3-amino-9-ethyl-carbazol (AEC, Sigma, St. Louis, MO, USA) and counterstaining was performed with hematoxylin.

MLH1: Monoclonal mouse anti-human MLH1-antibody (Zymed, Berlin, Germany) was used at a dilution of 1 : 80. Pretreatment was done by microwaving with target unmasking fluid (TUF, DAKO, Hamburg, Germany) for 30 min at high power. Incubation was performed overnight at room temperature, visualization was carried out using Envision+ peroxidase complex (DAKO, Hamburg, Germany) with AEC.

The staining pattern for both antibodies was nuclear. A negative staining reaction in tumor cells was only regarded as a loss of protein expression in the presence of a positive staining reaction in stromal

cells and lymphocytes which served as internal controls. Specificity of staining was verified by replacement of the primary antibody with the mouse IgG isotype (Southern Biotechnology Associates INC., Birmingham, GB) resulting in a negative staining reaction.

RESULTS

The 40 HNPCC index patients (31 Amsterdam positive, 9 Bethesda positive; 21 females, 19 males) had a mean age of 48.0 ± 13.2 years. The extended marker panel included one mono-A-run (BAT40), three dinucleotide repeats (D10S197, D13S153 and D18S58) and one tetra-nucleotide repeat (MYCL1).

The extended microsatellite analysis revealed 52 instabilities out of 200 analyses, in total.

As examples Fig. 1a/b and 2 a/b show the settings for the marker D18S58 and MYCL1 with a stable and instable status with additional bands in the tumor DNA, respectively. The distribution of stabilities and instabilities of the 5 microsatellites are given in Fig. 3. From the originally diagnosed 31 MSS and 9 MSI-L tumors an extended marker panel led to a reclassifification with 12 MSS (30%), 24 MSI-L (60%) and even 4 MSI-H (10%) tumors. All MSI-H tumors came from Amsterdam positive families.

The mean age of onset for CRCs was the lowest in the MSI-H group with 45.7 ± 9.6 years compared to 48.7 ± 14.3 and 49.0 ± 12.9 years in the MSI-L -and MSS group.

MSI-H-and MSI-L tumors were often localized in the proximal colon (50 and 52%), whereas MSS tumors were preferentially localized in the distal colon (77%) (Fig.4).

DISCUSSION

Following the recommendation of the International Collaborative Group (ICG on HNPCC) [6], we first examined the reference panel of five microsatellite markers in 249 patients with a familiy history of HNPCC. Among them we selected a group of 40 patients, 31 fulfilling the Amsterdam, 9 fulfilling the Bethesda criteria, who did not reveal neither the expected high graded microsatellite instability nor a mutation in the most commonly affected MMR genes MSH2 and MLH1.

To reconfirm the MSS status or to reveal putative instabilities in other than the recommended markers,

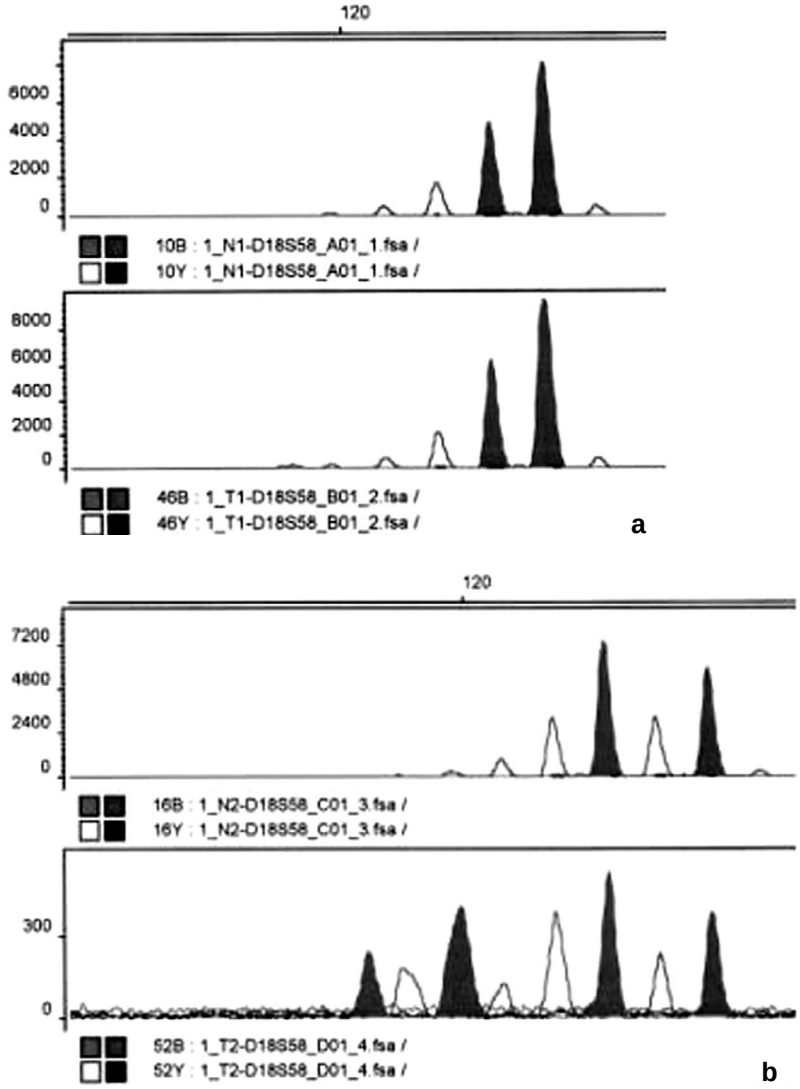

Fig. 1. Microsatellite analyses: Lanes above represent the normal DNA, lanes below the corresponding tumor DNA, respectively. *a*) D18S58 (dinucleotid repeat): stable; *b*) D18S58: additional bands in the tumor DNA, instable.

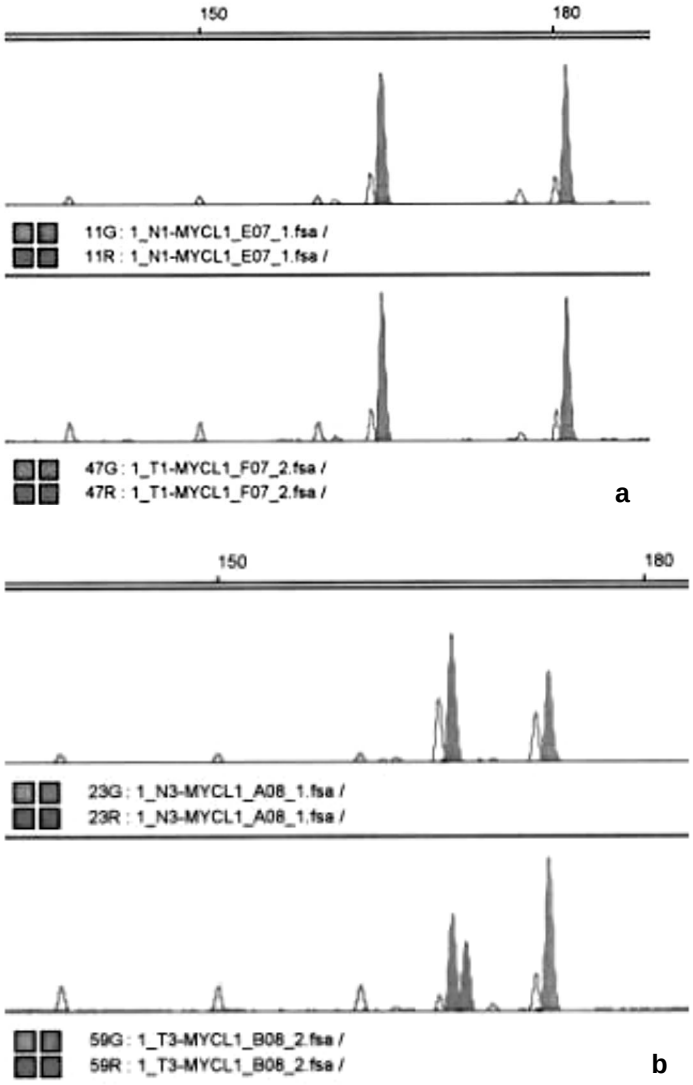

Fig. 2. Microsatellite analyses: Lanes above represent the normal DNA, lanes below the corresponding tumor DNA, respectively. *a*) MYCL1 (tetranucleotid): stable. *b*) MYCL1: an additional band in the tumor DNA, instable.

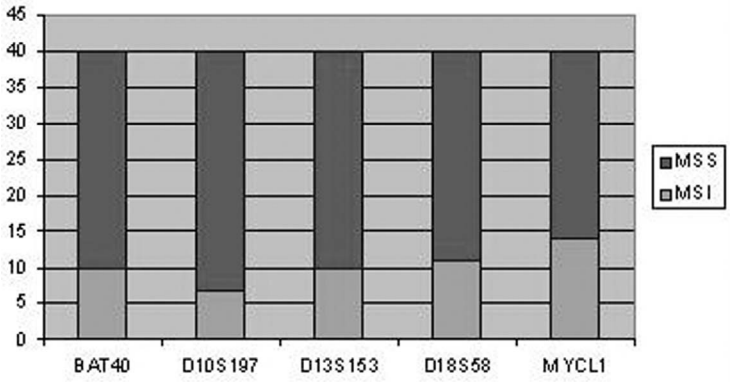

Fig.3. Distribution of instability (MSI) and stability (MSS) of each additional microsatellite marker among the 40 MMR gene mutation-negative HNPCC tumors.

we performed an extended microsatellite analysis with one mono-(BAT40), three di-(D10S197, D13S153, D18S58), and one tetranucleotide (MYCL1) repeat. These five microsatellites were chosen since they had been shown to be also useful in HNPCC screening [11]. Among them, especially MYCL1 has been shown to have a relatively high instability rate.

Due to the extended panel of five additional microsatellite markers 4 tumors now revealed an MSI-H phenotype, which, in combination with a positive family history, is a very strong sign for cancerogenesis following the MIN-pathway.

Furthermore, 24 tumors could be reclassified as MSI-L phenotype (60%). Interestingly, this group included 6 tumors (15%) with three instable markers. Since recently published studies qualified already tumors with 30% of instable markers as MSI-H tumors [15], the number of our detected MSI-H

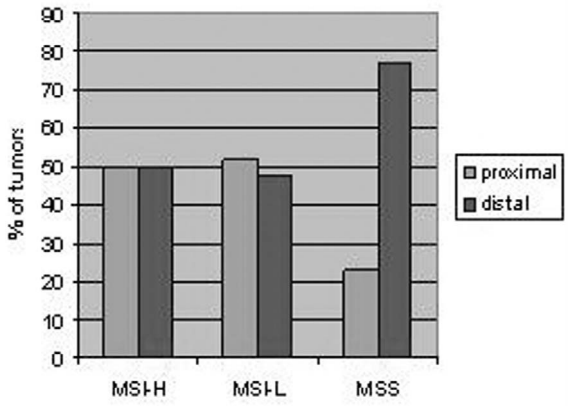

Fig. 4. The distribution of CRC localization in dependence on the reclassified MSI-status of the 40 mutation-negative HNPCC tumors.

phenotypes would increase to 10 cases in total (25%).

With the extended marker panel of microsatellites, 12 tumors were reconfirmed as MSS phenotypes (30%). Their preferential tumor localization in the distal colon hints to a similar biologic behavior to that of sporadic CRC, which are known to be predominantly localized in the distal colon. This supports data of recent comparative studies on HNPCC patients with and without detectable MMR gene mutations [16-19]. Among the mutation negative patients, the authors described a major subgroup with a later age of onset, an abundance of distal colorectal cancer and fewer HNPCC-related cancers. These tumors may follow the CIN- instead of the MIN-pathway, what we already supposed due to our own recent study on a smaller group of MMR gene mutation negative patients [12]. Maybe that this genetic reclassification influence the decision of the clinicians of whether to offer patients chemotherapy or not, since it is known that patients with instable tumors do not benefit from chemotherapy as well as patients with microsatellite stable tumors [15].

We conclude that MSS or MSI-L phenotypes of clinically diagnosed HNPCC tumors should not be considered as an exclusion criterion for complementary microsatellite analysis in general, above all if the patients fulfill the strict Amsterdam criteria, which are known to be the best predictive clinical features [20, 21]. Extended microsatellite analysis helps to identify "occult" MSI-H tumors, for whom an additional mutation analysis of other MMR genes (MSH6, PMS1, MLH3) would be ingenious.

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REFERENCES

1. Lynch HAT, Smyrk T, Lynch J. An update of HNPCC (Lynch Syndrome). Cancer Genet Cytogenet 1997;93:84-99.
2. Aaltonen LA, Peltomaki P, Leach FS et al. Clues to the pathogenesis of familial colorectal cancer. Science 1993;260:812-816.
3. Fishel R, Lescoe MK, Rao MRS et al. The human mutator gene homolog MSH2 and ist association with hereditary nonpolyposis colon cancer. Cell 1993;75:1027-1038.
4. Papadopoulos N, Nicolaides NC, Wie Y et al. Mutation of a mutL homolog in hereditary colon cancer. Science 1994;262:1625-1629.
5. Edelmann W, Yang K, Umar A et al. Mutation in the mismatch repair gene MSH6 causes cancer susceptibility. Cell 1997;91:467-468.
6. Boland CR, Thibodeau SN, Hamilton SR et al. A National Cancer Institute Workshop on microsatellite instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Research 1998;58:5248-5257.
7. Wu Y, Berends MJ, Mensink RG et al. Association of hereditary nonpolyposis colorectal cancer-related tumors displaying low microsatellite instability with MSH6 germline mutations. Am J Hum Genet 1999;65:1291-1298.
8. Wijnen J, Khan PM, Vasen H et al. Hereditary nonpolyposis colorecal cancer families not complying with the Amsterdam criteria show extremely low frequency of mismatch-repair-gene mutations. Am J Hum Genet 1997;61:329-335.
9. Vasen HFA, Mecklin JP, Meera KP et al. The International Collaborative Group on hereditary non-polyposis colorectal cancer. Dis Colon Rectum 1991; 34:424-425.
10. Koreth J, O´Leary J, McGee J. Microsatellites and PCR genomic analysis. J Pathol 1996; 178:239-248.
11. Dietmaier W, Wallinger S, Bocker T et al. Diagnostic microsatellite instability: definition and correlation with mismatch repair protein expression. Cancer Res 1997; 57:4749-4756.
12. Schiemann U, Müller-Koch Y, Gross M, Daum J, Lohse P, Baretton G, Muders M, Mussack T, Kopp R, Holinski-Feder E. Extended microsatellite analysis in microsatellite stable, MSH2 and MLH1 mutation-negative HNPCC patients: genetic reclassification and correlation with clinical features. Digestion 2004;69:166-176.
13. Holinski-Feder E., Müller-Koch Y., Friedl W. et al. DHPLC mutation analysis of the hereditary nonpolyposis colon cancer (HNPCC) genes hMLH1 and hMSH2. J Biochem Biophys Methods 2001;47:21-32.
14. Müller-Koch Y, Kopp R, Lohse P et al. Sixteen rare sequence variants of the hMLH1 and hMSH2 genes found in a cohort of 254 suspected HNPCC patients: mutations or polymorphism? Eur J Med Res 2001; 6:473-482.
15. Ribic CM, Sargent DJ, Moore MJ et al. Tumor microsatellite-instability status as a predictor of benefit from fluorouracil based adjuvant chemotherapy for colon cancer. N Engl J Med 2003; 349(3):247-257.
16. Bisgaard ML, Jager AC, Myrhoij T et al. Hereditary nonpolyposis colorectal cancer (HNPCC): phenotype-genotype correlation between patients with and without identified mutation. Hum Mutat 2002; 20:20-27.
17. Benatti P, Roncucci L, Ganazzi D et al. Clinical and biologic heterogeneity of hereditary nonpolyposis colorectal cancer. Int J Cancer 2001; 95:323-8.
18. Rovella V, Carrara S, Crucitti SC et al. Familial microsatellite-stable non-polyposis colorectal cancer: incidence and characteristics in a clinic-based population. Ann Oncol 2001;12:813-8.
19. Scott RJ, McPhilipps M, Meldrum CJ et al. Hereditary nonpolyposis colorectal cancer in 95 families: differences and similarities between mutation-positive and mutation-negative kindreds. Am J Hum Genet 2001; 68:118-127.

20. Wijnen J, Khan PM, Vasen H et al. Hereditary nonpolyposis colorectal cancer families not complying with the Amsterdam criteria show extremely low frequency of mismatch-repair-gene mutations. Am J Hum Genet 1997; 61:329-35.
21. Brassett C, Joyce JA, Frogatt NJ et al. Microsatellite instability in early onset and familial colorectal cancer. J Med Genet 1996; 33:981-5.

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