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GENETICS ANALYSIS OF MICROSATELLITE MARKERS IN PATIENTS FROM HEREDITARY NONPOLYPOSIS COLORECTAL CANCER (HNPCC) FAMILY

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Aim: **Microsatellite instability (MSI) is due to defective DNA mismatch repair (MMR) and is characteristic of hereditary nonpolyposis colorectal cancer (HNPCC) tumors. The role of MSI in familial predisposition to colorectal cancer was investigated in this study by both microsatellite analysis and mutation screening of the two major MMR genes *MLH1* and *MSH2* among familial cases.** ***Methods:*** **PCR-based microsatellite analysis was performed in blood obtained from 30 members from HNPCC families. Blood samples age matched healthy individuals (n = 28) served as control. MSI was studied at five loci containing single- or dinucleotide repeat sequences and mapping to different chromosomal locations: BAT-25 (at locus 4q12), BAT-26 (2p16), D2S123 (2p16-p21), D5S346 (5q21-q22) and D17S250 (17q11.2-q12).** ***Results:*** **MSI frequency was higher in member of HNPCC families [7/30 (23%)] than in control [3/28 (10.7%)] cases. Two *MLH1* and one *MSH2* mutations were identified in 7 MSI positive samples from HNPCC families. *MLH1/MSH2* mutations were only in MSI high samples detected.** ***Conclusion:*** **Genetic alterations seem to be a risk factor of colorectal cancer in subjects belonged to HNPCC families with high incidence of this cancer.**

Key Words**: microsatellite instability, *MLH1/MSH2* genes, hereditary nonpolyposis colorectal cancer.**

Hereditary nonpolyposis colorectal cancer (HNPCC) is one of the most common genetic diseases in the Western world [1]. HNPCC is often divided into two subgroups, type I Lynch syndrome and type II Lynch syndrome. Individuals with type I Lynch syndrome have a hereditary predisposition to colorectal cancer that is distinguished from sporadic colorectal cancer; the carcinomas in type I Lynch syndrome are often observed in the proximal colon. In families with type II Lynch syndrome, patients have an increased risk for cancers in certain tissues such as the uterus, ovary, breast, stomach, skin and larynx in addition to the colon [2, 3].

HNPCC is a dominantly inherited tendency to form cancers in the colon and rectum, and sometimes other organs, at a young age. This tendency is due to a defect in DNA repair capacity that results from a mutation in one of the genes coding for DNA repair enzymes [4, 5]. Such mutations produce unstable DNA, which is then more liable to accumulate the other genetic changes that promote carcinogenesis. The effect of these genetic abnormalities is to produce the clinical picture (phenotype) of HNPCC [6, 7].

Microsatellite instabilty (MSI) seems to be important in the development of various human cancers and was first detected in tumors from patients with HNPCC [8–10]. Microsatellite sequences are short repeated nucleotides sequences disseminated in whole genome in normal conditions. In Eucaryota genome there are repeated sequences consisted of one, two, three and four nucleotides. Over 90% studied till this moment microsatellite sequences from mononucleotides to tetranucleotides show polymorphism. Small deletions or expansions in tumor DNA, manifested as shifts in allelic electrophoretic mobility characterise microsatellite instability. Genetic instability is considered to be responsible for a rapid accumulation of somatic mutations in various tumor suppressor genes and oncogenes, thus playing an important role in the initiation and progression of malignant tumors [11, 12].

A total of 85–90% of HNPCC patients show MSI [13, 14], and this proportion is even higher in mutation–positive families, whereas only 10–15% of sporadic colorectal tumors do so [15, 16].

The aim of this study was to investigate whether the presence of MSI and *MLH1/MSH2* mutations in members from HNPCC family with high incidence of colorectal cancer may be a risk factor of this cancer appearance. This work was performed to test MSI analysis using the Bethesda panel of five markers [8]; two mononucleotide repeats (BAT25 and BAT26) and three dinucleotide repeats (D2S123, D5S346 and D17S250). Furthermore the mutations in *hMLH1* and *hMSH2* were sought in all patients whose tumors were microsatellite instability positive.

MATERIALS AND METHODS

Patients and DNA isolation. Blood was obtained from 30 members from HNPCC families at Department of Surgery at the Medical University in Lodz (Poland) between 2003–2004 (Table 1). All patients were asked to provide a blood sample for mutation analysis and complete a self–administered family history questionnaire to document the current ages, ages at death,

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Abbreviations used: HNPCC — hereditary nonpolyposis colorectal cancer; MSI — microsatellite instability; MMR — mismatch repair; PCR — polymerase chain reaction.

causes of death, and ages at diagnosis of any cancer and the types of cancer found in any member of their first–, second–, or third–degree relatives.

There were 16 males and 14 females and their mean age was 42 years (range: 24–80 years). Blood samples age matched healthy individuals (n = 28) served as control.

DNA was extracted from blood using commercially available QIAmp Kit (Qiagen GmbH, Hilden, Germany) DNA purification kit according to manufacturer's instruction.

Microsatellite analysis. DNA from members from HNPCC families and corresponding control DNA were analysed using a panel of the five microsatellite markers for mononucleotide and dinucleotide repeat sequences: BAT 25 (at locus 4q12), BAT 26 (2p16), D2S123 (2p16–p21), D5S346 (5q21–q22) and D17S250 (17q11.2–q12) [3]. All primer sequences were as reported in Genome DataBase (GDB, at: http://www.gdb.org). The PCR cycles for each marker are indicated in Table 2.

The PCR was carried out in a Perkin–Elmer/Gene Amp, PCR System 2400 thermal cycler. PCR amplification was performed in a final volume of 25 μl. The reaction mixture contained 5 ng genomic DNA, 0.2 μmol of each appropriate primer (ARK Scientific GmbH Biosystems, Darmstad, Germany), 2.5 mM $MgCl_2$, 1 mM dNTPs and 1 unit of Taq Polymerase (Qiagen GmbH, Hilden, Germany). PCR products were fractionated by denaturing electrophoresis in a 6% polyacrylamide gel

Table 1. The results of MSI and *hMLH1* and *hMSH2* mutation analysis in 30 investigated subjects from HNPCC family

Patient No	Age at diagnosis	Family history	MSI markers positive	MSI classification	*MLH1*	*MSH2*
1	29	Father prostate cancer, two paternal sisters breast and ovarian cancer, paternal grandfather prostate cancer, paternal grandmother lung cancer, mother colon cancer, maternal sister colon cancer	5/5	MSI–H	1946delC	No
2	44	Paternal brother lung cancer, maternal brother colon cancer, maternal grandfather colon cancer	0/5	MSS	No	No
3	46	Paternal sister breast cancer, paternal grandfather colon cancer, paternal grandmother colon cancer, maternal brother colon cancer	0/5	MSS	No	No
4	51	Paternal brother lung cancer, maternal brother colon cancer, maternal grandfather colon cancer	0/5	MSS	No	No
5	54	Mother colon cancer, maternal sisters colon cancer, maternal grandfather prostate cancer, maternal grandmother colon cancer	0/5	MSS	No	No
6	48	Mother colon cancer, maternal brother brain tumor, maternal grandfather colon cancer	0/5	MSS	No	No
7	51	Brother colon cancer, father colon cancer, paternal brother colon cancer, paternal grandmother colon cancer	4/5	MSI–H	1946delC	GGC (Gly) to GAC (Asp) at codon 322
8	55	Father colon cancer, paternal brother colon cancer, paternal grandmothers colon cancer	3/5	MSI–H	GAG (Glu) to GGG(Gly) mutation at codon 578	No
9	36	Mother colon cancer, maternal grandmother breast cancer, maternal great–grandmother colon cancer	0/5	MSS	No	No
10	24	Maternal brother colon cancer, maternal grandmother colon cancer	0/5	MSS	No	No
11	29	Paternal brother colon cancer, paternal grandmother colon cancer, maternal grandmother brain tumor	0/5	MSS	No	No
12	24	Father colon cancer, paternal grandmother colon cancer	2/5	MSI–H	1946delC	No
13	52	Two brothers colon cancer, mother colon cancer, maternal sister colon cancer, maternal grandfather prostate cancer, maternal grandmother colon cancer	0/5	MSS	No	No
14	48	Brother colon cancer, mother colon cancer, maternal sister colon cancer, maternal grandfather prostate cancer, maternal grandmother colon cancer	0/5	MSS	No	No
15	24	Father colon cancer, paternal grandfather colon cancer, maternal brother colon cancer, maternal grandmother colon cancer	2/5	MSI–H	GAG (Glu) to GGG(Gly) mutation at codon 578	No
16	46	Brother colon cancer, mother colon cancer, maternal brother colon cancer, maternal grandmother colon cancer	1/5	MSI–L	No	No
17	25	Father colon cancer, paternal grandmother colon cancer	0/5	MSS	No	No
18	48	Mother colon cancer, maternal brother colon cancer, maternal grandmother colon cancer	0/5	MSS	No	No
19	46	Sisters breast cancer, mother renal cancer, maternal sister colon cancer, maternal grandfather colon cancer	0/5	MSS	No	No
20	48	Father colon cancer, paternal brother colon cancer, paternal sister colon cancer, maternal grandfather lung cancer	0/5	MSS	No	No
21	26	Paternal grandfather colon cancer, maternal grandmother colon cancer	0/5	MSS	No	No
22	60	Sister breast cancer, mother ovarian cancer, maternal sister ovarian cancer, daughter colon cancer	0/5	MSS	No	No
23	36	Mother colon cancer, maternal brother bone sarcoma, maternal sister colon cancer	0/5	MSS	No	No
24	26	Father colon cancer, paternal grandfather colon cancer, maternal sister breast cancer	0/5	MSS	No	No
25	55	Brother colon cancer, father colon cancer	0/5	MSS	No	No
26	46	Brother colon cancer, father colon cancer	2/5	MSI–H	No	No
27	26	Paternal brother colon cancer, paternal grandfather colon cancer	0/5	MSS	No	No
28	80	Brother colon cancer, father colon cancer, paternal brother colon cancer, paternal sister leukemia	0/5	MSS	No	No
29	39	Mother colon cancer, maternal sister colon cancer	0/5	MSS	No	No
30	46	Sisters breast cancer, mother colon cancer, maternal sister colon cancer, maternal grandfather colon cancer	0/5	MSS	No	No

MSI–H – MSI–high; MSS – MS–stable; MSI–L – MSI–low

Table 2. Primer sequences

Marker	Primer sequences	PCR cycles
BAT25	TCG CCT CCA AGA ATG TAA GT TCT GGA TTT TAA CTA TGG CTC	28 cycles of 95 °C for 1 min, 56 °C for 45 s, 72 °C for 45 s
BAT26	TGA CTA CTT TTG ACT TCA GCC AAC CAT TCA ACA TTT TTA ACC	32 cycles of 95 °C for 45 s, 55 °C for 1 min, 72 °C for 30 s
D2S123	AAA CAG GAT GCC TGC CTT TA GGA CTT TCC ACC TAT GGG AC	35 cycles of 95 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s
D5S346	ACT CAC TCT AGT GAT AAA TCG GG AGC AGA TAA GAC AAG TAT TAC TAG	30 cycles of 95 °C for 1 min, 57 °C for 45 s, 72 °C for 45 s
D17S250	GGA AGA ATC AAA TAG ACA AT GCT GGC CAT ATA TAT ATT TAA ACC	35 cycles of 95 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s

and visualised by silver staining. Patients whose DNA showed alleles that were not present in the corresponding control DNA were classified as MSI positive.

DNA analysis. Mutation analysis of the *MLH1* and *MSH2* genes was performed in DNA from peripheral blood lymphocytes obtained from all patients. All exons of the *MSH2* and *MLH1* genes were screened by direct sequencing of genomic PCR products. The PCR products were sequenced using the SequiTherm EXCEL ™ II DNA Sequencing Kit (EPICENTRE®, Madison, USA).

Statistical analysis. For statistical analysis, the χ^2 test was performed, $p < 0.05$ was considered significant.

RESULTS

A sample was classified as MSI–high (MSI–H) if two or more markers showed instability, MS–stable (MSS) if no instability was noted, and MSI–low (MSI–L) if a single marker revealed novel bands compared with the corresponding control DNA.

A total of 30 subjects from HNPCC families were successfully analysed for MSI using the Bethesda panel of five microsatellite markers. A total of 5 of 30 patients (16.7%) had previously shown MSI when analyzed using BAT26 alone (Table 3). When the five Bethesda markers were used, all 5 appeared as MSI–H. A total of 7 of 30 patients (23.3%) were classified as MSI with the Bethesda panel; 6 (20%) patients were classified as MSI–H, and 1 patient (3.3%) was classified as MSI–L.

MSI was determined in DNA samples from HNPCC family compared as 28 control samples. It can be seen from the Table 3 that the presence of MSI in members from HNPCC families was higher than in control samples ($p < 0.05$).

Mutation analysis of the *MLH1* and *MSH2* genes was performed in 30 members from HNPCC families. Out of the 7 MSI positive samples three mutations were found. These were a 1946delC (truncating mutation) and germline GAG (Glu) to GGG(Gly) mutation at codon 578 of the *MLH1* gene and germline GGC (Gly) to GAC (Asp) at codon 322 of the *MSH2*. The samples with these mutations were MSI high.

DISCUSSION

HNPCC is an autosomal dominant disorder clinically defined by the revised Amsterdam criteria [16]. In HNPCC subjects the median age at development of colorectal cancer is about 44 and 47 years. Predisposition to HNPCC is the result of germline mutations in the mismatch repair genes [17–19].

The number of genes involved in HNPCC and the number of possible mutations in those genes make DNA analysis for mutation detection a time–consuming and expensive process. Reportedly, affected members in HNPCC families have inherited a mutated allele of either one of the five mismatch repair (*MMR*) genes, *MSH*2 [20], *MLH*1 [21], *PMS*1 [22], *PMS*2 [22] and *MSH*6 [23]. Although the proportion of HNPCC attributable to each of these genes remains unclear, mutation of *MLH1* or *MSH*2 gene is considered to be the most common cause; each accounts for 30–40% of germline mutations so far reported in HNPCC families [24–27].

In light of substantial evidence that the appearance of colon cancer can be associated with MSI it seems reasonable to check a possible correlation between MSI and high–risk of colorectal cancer in patients from HNPCC family. In this work conducted on 30 members from HNPCC family with high incidence of colon cancer we find correlation between microsatellite instability and risk of colorectal cancer appearance.

In an attempt to determine if patients from HNPCC family are under MSI, genomic DNA was extracted from control and patients blood diagnosed at the Department of Surgery at the Medical University in Lodz. In this study a reference panel of five polymorphic markers (two mononucleotide repeats and three dinucleotide repeats) was used.

In the group of 30 members from HNPCC family, microsatellite instability was found in 7 (23.3%). These 7 cases plus 23 other subjects whose tumors were MSI negative were studied for *MLH1* and *MSH2* somatic and germline mutations. Germline mutation in *MLH1* was found in two investigated cases (6.6%) and in *MSH2* in one case (3.3%). These mutations in members from HNPCC families with high incidence of colon cancer suggested their potential role in appearance of this cancer. Moreover the analysis showed that MSI present most frequently in members from HNPCC family with high frequency of colon cancer compared as control. It is suggest that MSI may be associated with high risk of colon cancer in member from these families.

Table. 3. Number of microsatellite instability cases in HNPCC family compared to control

Subjects from HNPCC families (n = 30)						
	MSI with the Bethesda markers					
MSI with BAT26 alone	0 positive markers	1 positive marker	2 positive markers	3 positive markers	4 positive markers	5 positive markers
BAT26 positive (n = 5)	No	No	n = 2	n = 1	n = 1	n = 1
BAT26 negative (n = 25)	n = 23	n = 1	n =1	No	No	No
Control (n = 28)						
	MSI with the Bethesda markers					
MSI with BAT26 alone	0 positive markers	1 positive marker	2 positive markers	3 positive markers	4 positive markers	5 positive markers
BAT26 positive (n = 1)	No	No	n = 1	No	No	No
BAT26 negative (n = 27)	n = 25	n = 2	No	No	No	No

In present work we confirm earlier observations [6, 7, 28] that in patients from HNPCC family mutations in *MLH1* and *MSH2* genes are associated with the presence of MSI–high.

The present study suggests that MSI seem to be associated with different tumorigenic pathways. The genome–wide MSI may be correlated to the existence of pathogenetic mechanisms inducing progressive accumulation of sequence errors and providing a selective advantage during malignant evolution. Moreover the relation between the presence of *MLH1* and *MSH2* mutations and MSI in members from HNPCC family provides a motive for the use of microsatellite instability in these cases as a pre–screening method for detection group of high risk of colon cancer appearance. Further studies, conducted on a larger population, are required to clarify this point.

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ГЕНЕТИЧЕСКИЙ АНАЛИЗ МИКРОСАТЕЛЛИТНЫХ МАРКЕРОВ У ПАЦИЕНТОВ С НАСЛЕДСТВЕННЫМ НЕПОЛИПОЗНЫМ КОЛОРЕКТАЛЬНЫМ РАКОМ

Цель: **микросателлитная нестабильность (MSI) связана с неправильным восстановлением поврежденной ДНК (MMR), что характерно для наследственного неполипозного колоректального рака. Роль MSI в наследственной предрасположенности к колоректальному раку изучалась методами микросателлитного анализа и мутационного скрининга двух главных генов MMR — *MLH1* и *MSH2*. *Методы:* микросателлитный анализ, основанный на PCR, проводили с использованием образцов крови 30 человек с семейной историей неполипозного колоректального рака (ННПКР). В качестве контроля были использованы образцы крови здоровых доноров соответствующей возрастной группы (n = 28). MSI исследовали в 5 локусах, содержащих моно- и динуклеотидные повторы и картированные в разных локализациях: BAT-25 (в локусе 4q12), BAT-26 (2p16), D2S123 (2p16-p21), D5S346 (5q21-q22) и D17S250 (17q11.2-q12). *Результаты:* частота MSI была выше у членов семей со случаями ННПКР [7/30 (23%) случаев] в сравнении с контрольной группой [3/28 (10,7%) случаев]. Две мутации гена *MLH1* и одна — *MSH2* были идентифицированы в 7 MSI-позитивных образцах крови членов семей с ННПКР в семье. Мутации генов *MLH1/MSH2* были выявлены только в образцах с высокой частотой MSI. *Выводы:* генетические изменения могут быть факторами риска при колоректальном раке у членов семей с историей ННПКР.**

***Ключевые слова*: микросателлитная нестабильность, гены *MLH1/MSH2*, наследственный неполипозный колоректальный рак.**