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Analysis of Microsatellite Instability and BRCA1 Mutations in Patients from Hereditary Nonpolyposis Colorectal Cancer (HNPCC) Family*

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Susceptibility to colorectal cancer appears to be linked to germ-line mutations in genes causing various familial cancer syndromes. The objectives of this study were to determine microsatellite instability and the frequency of BRCA1 germ-line mutations in patients with family history of cancer. The study population consisted of 30 patients from HNPCC family. Patients completed a family history questionnaire and provided blood for mutation analysis. Seven out of 30 investigated samples (23%) were found to be MSI-positive, 6 MSI-high and 1 MSI-low. Ex20insC and ExII17delA mutations of BRCA1 gene were identified in MSI-positive samples from HNPCC families. In the present study one Ex20insC mutation and two ExII17delA mutations were detected only in MSI-high samples. Genetic alterations seem to be a risk factor of colorectal cancer in subjects belonging to HNPCC families with high incidence of this cancer. The lack of detectable germ-line mutations in most cases suggests that there are probably additional, as yet unidentified genes predisposing to this disease.

Introduction

Hereditary Nonpolyposis Colorectal Cancer (HNPCC) is one of the most common genetic diseases in the Western world [15, 16]. 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 distinguishable 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 [4, 11].

HNPCC is a dominantly inherited tendency to form cancers in the colon and rectum, and sometimes in the 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 [20, 25]. 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 [14, 23].

Microsatellite instability (MSI) seems to be important in the development of various human cancers and was first detected in tumors from patients with HNPCC [17, 20, 26]. The dysfunction of the DNA mismatch repair (MMR) system causes genetic instability, and this instability has been demonstrated in tumors of HNPCC patients as frequent alterations at loci containing short, repetitive sequences, referred to as microsatellite instability and or replication errors [1, 2, 10].

A total of 85–90% of HNPCC patients show MSI [1, 2, 18], and this proportion is even higher in mutation-positive families, whereas only 10–15% of sporadic colorectal tumors do so [5, 27].

The aim of this study was to investigate whether the presence of MSI and BRCA1 gene mutations in members from HNPCC family with high incidence of colorectal cancer may be a risk factor of this cancer appearance. Studies have shown that hereditary breast and ovarian cancer may be associated with mutations in a number of tumor suppressor genes, mainly BRCA1 and BRCA2. Mutations in

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BRCA1 and/or BRCA2 profoundly increase the risk of breast and ovarian cancers, but it is unclear whether mutations in these genes increase the risk of colorectal cancer.

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 BRCA1 were sought in all patients whose tumors were microsatellite instability positive.

Material and Methods

Patients and DNA isolation

Blood was obtained from 30 members from HNPCC families at Department of Surgery at the Medical University in Łódź between 2003 and 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 age, age at death, causes of death, and age 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 from age matched healthy individuals (n=28) served as a 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 analyzed using a panel of the five microsatellite markers for mononucleotide and dinucleotide repeat sequences: BAT25 (at locus 4q12), BAT26 (2p16), D2S123 (2p16-p21), D5S346 (5q21-q22) and D17S250 (17q11.2-q12) [4]. All primer sequences were as reported in Genome DataBase (GDB, at: http://www.gdb.org). The PCR cycles for each marker are summarized in the 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 25µl. The reaction mixture contained 5 ng genomic DNA, 0.2 µmol of each appropriate primer (ARK Scientific GmbH Biosystems, Darmstad, Germany), 2.5 mM MgCl2, 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 and visualized 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 BRCA1 gene was performed in DNA from peripheral blood lymphocytes obtained from all patients using commercially available kit according to manufacturer's instruction (Pomeranian Medical University, Szczecin, Poland) (Fig. 1).

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.

All the patients from HNPCC families were successfully analyzed for MSI using the Bethesda panel of five microsatellite markers (Table 3). MSI was determined in the blood from 30 subjects and 28 control samples. Seven out of the 30 cases tested (23%) were found to be MSI-positive, 6 MSI-high and 1 MSI-low. It can be seen from the Table 3, that there were significant differences between patients and control. The presence of MSI in patients from HNPCC families was higher than in control samples ($p<0.05$).

Mutation analysis of BRCA1 gene was performed in members from HNPCC families. In the group of 30 samples three mutations were found. These were: one Ex20insC mutation and two ExII17delA mutations of the BRCA1 gene. The samples with these mutations were MSI-high (Table 1).

Discussion

Hereditary Nonpolyposis Colorectal Cancer is an autosomal dominant disorder clinically defined by the revised Amsterdam criteria [27]. In HNPCC subjects the median age at development of colorectal cancer is ca. 44–47 years. Predisposition to HNPCC is a result of germ-line mutations in the mismatch repair genes [7, 16, 22].

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 genes, *MSH*2 [6], *MLH*1

TABLE 1
The results of MSI and BRCA1 mutation analysis in 30 patients from HNPCC family

Patient No	Age at diagnosis	Family history of cancers	MSI markers positive	MSI classification	BRCA1 Ex20insC	BRCA1 ExII17delA
1.	29	father prostate, two paternal sisters breast and ovarian cancer, paternal grandfather prostate, paternal grandmother lung cancer, mother colon cancer, maternal sister colon cancer	5/5	MSI-H	+	-
2.	44	paternal brother lung cancer, maternal brother colon cancer, maternal grandfather colon cancer	0/5	MSS	-	-
3.	46	paternal sister breast cancer, paternal grandfather colon cancer, paternal grandmother colon cancer, maternal brother colon cancer	0/5	MSS	-	-
4.	51	paternal brother lung cancer, maternal brother colon cancer, maternal grandfather colon cancer	0/5	MSS	-	-
5.	54	mother colon cancer, maternal sisters colon cancer, maternal grandfather prostate, maternal grandmother colon cancer	0/5	MSS	-	-
6.	48	mother colon cancer, maternal brother brain tumor, maternal grandfather colon cancer	0/5	MSS	-	-
7.	51	brother colon cancer, father colon cancer, paternal brother colon cancer, paternal grandmother colon cancer	4/5	MSI-H	-	+
8.	55	father colon cancer, paternal brother colon cancer, paternal grandmother colon cancer	3/5	MSI-H	-	-
9.	36	mother colon cancer, maternal grandmother breast cancer, maternal great-grandmother colon cancer	0/5	MSS	-	-
10.	24	maternal brother colon cancer, maternal grandmother colon cancer	0/5	MSS	-	-
11.	29	paternal brother colon cancer, paternal grandmother colon cancer, maternal grandmother brain tumor	0/5	MSS	-	-
12.	24	father colon cancer, paternal grandmother colon cancer	2/5	MSI-H	-	-
13.	52	two brothers colon cancer, mother colon cancer, maternal sister colon cancer, maternal grandfather prostate, maternal grandmother colon cancer	0/5	MSS	-	-
14.	48	brother colon cancer, mother colon cancer, maternal sister colon cancer, maternal grandfather prostate, maternal grandmother colon cancer	0/5	MSS	-	-
15.	24	father colon cancer, paternal grandfather colon cancer, maternal brother colon cancer, maternal grandmother colon cancer	2/5	MSI-H	-	+
16.	46	brother colon cancer, mother colon cancer, maternal brother colon cancer, maternal grandmother colon cancer	1/5	MSI-L	-	-
17.	25	father colon cancer, paternal grandmother colon cancer	0/5	MSS	-	-
18.	48	mother colon cancer, maternal brother colon cancer, maternal grandmother colon cancer	0/5	MSS	-	-
19.	46	sisters breast cancer, mother renal cancer, maternal sister colon cancer, maternal grandfather colon cancer	0/5	MSS	-	-
20.	48	father colon cancer, paternal brother colon cancer, paternal sister colon cancer, maternal grandfather lung cancer	0/5	MSS	-	-
21.	26	paternal grandfather colon cancer, maternal grandmother colon cancer	0/5	MSS	-	-

22.	60	sister breast cancer, mother ovarian cancer, maternal sister ovarian cancer, daughter colon cancer	0/5	MSS	-	-
23.	36	mother colon cancer, maternal brother bone, maternal sister colon cancer	0/5	MSS	-	-
24.	26	father colon cancer, paternal grandfather colon cancer, maternal sister breast cancer	0/5	MSS	-	-
25.	55	brother colon cancer, father colon cancer	0/5	MSS	-	-
26.	46	brother colon cancer, father colon cancer	2/5	MSI-H	-	-
27.	26	paternal brother colon cancer, paternal grandfather colon cancer	0/5	MSS	-	-
28.	80	brother colon cancer, father colon cancer, paternal brother colon cancer, paternal sister leukemia	0/5	MSS	-	-
29.	39	mother colon cancer, maternal sister colon cancer	0/5	MSS	-	-
30.	46	sisters breast cancer, mother colon cancer, maternal sister colon cancer, maternal grandfather colon cancer	0/5	MSS	-	-

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

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

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

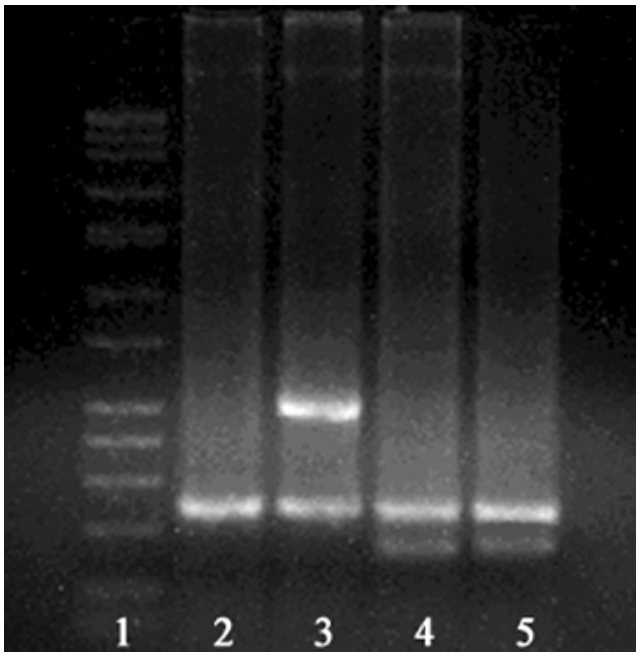

Fig. 1. A typical result of polymerase chain reaction performed with a fragment of the BRCA1 gene and analyzed by 2% agarose gel electrophoresis, stained with ethidium bromide and viewed under ultraviolet light. Lanes 1 - molecular weight markers, 50-2000 bp (Sigma, St. Louis, USA), lanes 2 - display the product of amplification without mutation, lanes 3 - display the product of amplification with the primer pair specific for the ExII17delA mutations, lanes 4 - display the product of amplification with the primer pair specific for Ex20insC mutations, lanes 5 - PCR control after restriction endonuclease *Ava*II digestion.

[19], *PMS*1 [3], *PMS*2 [3] and *MSH*6 [13]. Although the proportion of HNPCC attributable to each of these genes remains unclear, mutation of *MLH*1 or *MSH*2 gene is considered to be the most common cause; each accounts for 30–40% of germ-line mutations so far reported in HNPCC families [8, 9, 23, 24].

In the 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 including 30 members from HNPCC family with high incidence of colon cancer we found a correlation between microsatellite instability and the risk of colorectal cancer development.

In an attempt to determine if patients from HNPCC family present with MSI, genomic DNA was extracted from the controls and patients' blood diagnosed at the Department of Surgery, Medical University in Łódź. 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%). These 7 cases plus 23 other subjects whose tumors were MSI-negative were studied for BRCA1 germ-line mutations. Germ-line mutation in BRCA1 was found in three cases. These mutations in members from HNPCC families with high incidence of colon cancer suggested their potential role in the development of this cancer. A lack of detectable germ-line mutations in most cases suggests that there are probably additional, as yet unidentified genes predisposing to this disease.

Moreover, the analysis showed that MSI occur more frequently in members from HNPCC family with high frequency of colon cancer as compared to control. It suggests that MSI may be associated with high risk of colon cancer in members from these families.

In the present work we confirm previous observations [14, 12, 21], that in the patients with HNPCC the high frequency of MSI is observed.

The present study suggests that MSI seems 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, a relation between the presence of BRCA1 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 of a group with high risk of colon cancer development. Further studies, conducted on a larger population, are required to clarify this point.

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