Hereditary non polyposis colorectal cancer in a random sample of colorectal cancer patients

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Introduction

Colorectal cancer is one of the most frequent: it is the second most frequent in men and women, just after lung cancer and breast cancer, respectively. A significant number of people are at high risk of developing colorectal cancer if they carry germ line mutations. Molecular genetics promises to be the key for identification of the population at high risk of developing colorectal cancer. Genetic testing could be clinically used in the future to diagnose sporadic and hereditary colorectal cancer.

Mutations cause uncontrolled multiplication and the uncontrolled growth of mutated cells within the body. Mutations of control genes (control the cell cycle) cause genome instability. The most frequent mutation is of the p53 gene – a guardian of the...
genome. p53 protects DNA by blocking cell proliferation, stimulating DNA repair and promoting apoptosis. Neoplastic growth is inevitable without apoptosis (1).

All cancers are genetically caused. Development of cancer is the result of mutations in tumor suppressor genes, activation of oncogenes and mutations in DNA repair genes (MMR). Genetic pathways of cancer pathogenesis (2) are: the loss of heterozygosity (LOH) and defects in mismatch repair (MMR) genes.

Loss of heterozygosity is the complete loss of function of two corresponding genes (alleles)-deletion. The most frequent areas are 17p and 18 where tumor suppressor genes are located.

Defects in mismatch repair (MMR) genes are changes in short sequences of bases that are repeated and are found in the entire genome of cancer DNA, which is damaged in microsatellite regions-microsatellite instability (MSI). This is formation of new alleles at microsatellite loci in tumor DNA.

Hereditary cancers are microsatellite instability (MSI) in 90% of cases, and 10% of sporadic cancers are MSI. MSI causes an expression of transformed tumor growth factor on the cell surface (3).

According to the hereditary influence, cancers are classified as: sporadic colorectal cancer (60%), familial (30%) and hereditary (10%). There is average, increased and high risk for development of colorectal cancer.

Hereditary nonpolyposis colorectal cancer (HNPCC) is a condition caused by an alteration in one or more of the MMR genes. In around 50% of cases there is a chance of passing the causative gene to children. Carriers of genetic mutations have an 80% chance of developing cancer. This condition should be suspected when the Amsterdam II or the Bethesda-modified criteria are present (4).

Amsterdam criteria were established for diagnosing HNPCC (5); there should be at least three relatives with an HNPCC – associated cancer (colorectal cancer, cancer of the endometrium or ovarium, stomach, small bowel, pancreas, biliary tract, ureter or renal pelvis and brain). All the following criteria should be present: one should be a first degree relative of the other two; at least two successive generations should be affected; at least one cancer should be diagnosed before age 50; FAP should be excluded in the colorectal cancer case (if present); tumors should be verified by pathological.

Bethesda Guidelines were developed in 1996 and revised in 2000, in which criteria for the identification of colorectal tumors that should be tested for MSI were present (6): those who meet the Amsterdam criteria should be tested for MSI.

Cancers which are MSI should be tested by sequencing on germ line mutations. Genetic testing using gene sequencing of at least MSH2 and MLH1 that account for approximately 70% of HNPCC cases is the most sensitive and should be considered for affected individuals in families meeting the criteria and first-degree adult relatives of those with known mutations.

The latest genetic information, in future could be clinically significant in the prevention of colorectal cancer, pre-symptomatic cancer diagnosis, selection of patients for the most suitable treatment and evaluation of the malign potential (7).

Genetic therapy will probably be used in the future as the most optimal type of the therapeutic treatment.

There is no colorectal cancer registry in Bosnia and Herzegovina, but there is registry in the Tuzla county with 500,000 inhabitants. Until now colorectal cancer has only been diagnosed as sporadic. The aim of this research was to select individuals and families who should be screened for genetic testing for HNPCC and to diagnose hereditary colorectal cancer.
Material and Methods

Material

This prospective study was undertaken in the period from January to December 2000, in the Clinic of Internal Diseases, Surgery Clinic, and Department of Pathology in University clinical center Tuzla, Institute for Biochemistry, Medical School of Ljubljana, and Institute of Biochemistry, University of Oxford. The sample involved 40 patients with sporadic colorectal cancer. During this year in the Endoscopy Unit 152 colorectal cancers were colonoscopically diagnosed and 1200 colonoscopy were performed. In this sample there were 7.8% colorectal cancers found in the total of 1200 colonoscopy procedures. The sample size was limited to 40 colorectal cancers due to lack of resources, however we believe that this size is sufficient for the benefit of this study. The first page of the registration chart was reserved for personal data and an accurate genealogical tree, in which the main causes of morbidity and mortality of first degree relatives were recorded. Familial disease history analysis gave the data on colorectal cancer presence, adenoma, or other cancer in the family. The Amsterdam Criteria and Bethesda Criteria were used for genetic tests and HNPCC was diagnosed in the patients with previously diagnosed sporadic colorectal cancer.

Sporadic cancer is characterized by genetic mutations existing just in the cancer cell populations. In hereditary colorectal cancer genetic mutations exist in the cancer cell population, in surrounding tissue and peripheral blood. So, in this group of patients the control group are cells of surrounding healthy tissue.

Genetic tests of cancer tissue were undertaken and surrounding tissue was tested. The kind and frequency of genetic mutations characteristic for our tested group were identified.

Linkage analysis was performed to identify the LOH, as well as homozygosity for tumor suppressor genes. Genetic tests for microsatellite instability mismatch repair gene with identical microsatellite markers were carried out. In this way the most effective molecular screening for HNPCC was identified.

Methods

Genetic tests were performed by molecular biology methods. DNA isolation was performed from cancer tissue and the surrounding healthy tissue.

Genomic DNA isolation was performed on deparaffinization tissue sections as on cell proteolises of tissue by proteinase K and was checked by spectrophotometer and it ranged from 100-200 ng per sample (8).

Polymerase chain reaction (PCR): Chain DNA synthesis reaction by means of polymerase (PCR) is a method by which a specific DNA cut may be multiplied into a huge number of copies. A mixture of genome DNA with the gene that we want to multiply, individual nucleotides: a pair of initial oligonucleotides, Taq polymerase and Mg+ with optimal ph, should be made for PCR reaction. The PCR conditions were as follows: after initial 2 min denaturation at 94ºC, 30 amplification cycles were performed, each consisting of 10 s steps at 94ºC, 30 s steps at 55ºC, and a 30 s elongation step at 72º C. Amplification was completed with a final incubation step at 72ºC for 7 min. For separating of amplified PCR products, we used an automatic sequencer 310 ABI PRISM, Genetic Analyzer 310 (Perkin Elmer), which enable separating and quantification of DNK fragments according to the principle of capillary electrophoresis. PCR was performed using a PCR Thermocycler 9600 (Perkin-Elmer).

Fluorescent PCR is the method of wide usage in tumor detection, particularly im-
Fluorescent PCR of microsatellite markers: mononucleotides and dinucleotides markers were used for detection of microsatellite instability on MMR genes. In the group of mononucleotides markers BAT 25 were used (for microsatellite locuses on chromosome 4q12), BAT 26 (chromosome 2), and BAT 40 (chromosome 1P13.1). In the group of dinucleotide markers DS 123 were used (chromosome 2p16), and TP 53 (chromosome 17p13.1). Initial nucleotides in the marker BAT 25 were marked with HEX fluorescent dye. Predenaturation was done for each locus at 90°C, the temperature of annealing of primers and cycles number, the reaction of extensions of oligonucleotides. Conditions for amplification in a Master mix final volume of 25μl was: 2,5 μl 10 % puffer; 1μl DNA (5-10 ng); 2 μl (25 Mm) mGcl2; per 0,5 μl (2,5 mM) of each dNTP-a; 0,1 μl (0,5U) AmpliTaq Gold polymerase (Perkin-Elmer); 1 μl for-ward primer (12,5 pmol) and 1 μl reverse primer (12,5 pmol) by each pair of initial oligonucleotide for each microsatellite marker, and until 25 μl volumes of sterile water

Multiplex PCR: it is used for more prompt detection of loss of heterozygosity (LOH) of tumor suppressor genes. For Fluorescent detection LOH intragenic markers for the following tumor suppressor genes were used: NM 23 (chromosome 17p 21.3), p 53 (chromosome 17p 13.1), chromosome 5q 21-21), RB (chromosome 13q 14.1-q14.2), DCC1 and DCC2 with HEX. Initial oligonucleotides markers were marked by fluorescent dye for APC and RB1 with TET, markers for NM 23, p 53 and DCC1 with 6-FAM and DCC2 with HEX. Initial oligonucleotides of the chosen markers were multiplied for the locuses: NM 23, p 53, APC, RB1, and in the second, so called duplex reaction, initial oligonucleotides were multiplied for the locuses DCC1 and DCC2. The quality of multiplication and presence of possible contamination of PCR products was checked on 2% agar-gel.

Capillary electrophoresis on automatic sequenator: for separation of amplified products automatic sequenator 310 Genetic Analyzer was used. It enables fragmentation and quantification of DNK fragments by the principal of capillary electrophoresis. For microsatellite analysis and analysis of loss of heterozygosity we used fluorochromes: 6-FAM, TET, HEX (that marked 5 end) and GS TAMRA standards. Each color reflects the strongest fluorescence on different wavelengths. Gene Scan Tamra 500 was used for the analysis of the fragment’s size ranging from 35 to 500 bp.

Microsatellite analysis: fluorescent peaks are detected by means of software and shown on anelectrophoregram. Each fluorescent peak is automatically quantified according to the size of basis, height, and in the peak field. All the tests were repeated twice in order to confirm the obtained results.

RER analysis: each sample that had a new allele appearance in the tumor tissue in comparison to the corresponding normal tissue of the sample, represents microsatellite instability for the given marker.

LOH analysis: heterozygosity loss was calculated by mathematics (9). For heterozygosity cases the ratio of allele was calculated for each pair of normal and tumor tissue by the formula: T1:T2/N1:N2, where T1 and N1 values of the fields of shorter allele length, and T2 and N2 fields of longer allele fields for tumorous and healthy samples. In the cases where the allele ratio was above 1.00 the conversion 1 (T1:T2/N1:N2) was used. The result ranged from 0.00-1.00. If the result was under 0.50, significant heterozygosity loss of the longer allele was shown. Homozygous locuses were not taken for the analysis of LOH.
Colonoscopy is an endoscopic procedure made by videocolonoscope Olympus and used for diagnosing colorectal cancers, and for taking a sample for pathohistological analysis. For each of the patients three samples were taken from the cancer and two from healthy tissue 5 cm away from the cancer (control).

Results

Forty patients with sporadic colorectal cancers were diagnosed using endoscopic, surgical and pathohistologically methods. Family history indicated that 7 patients had family members with colorectal cancer or other HNPCC related cancers that were younger than 50 years of age. Amsterdam criteria were used to study these patients, and a genealogical family tree was drawn up for each patient.

By analyzing data according to the Amsterdam criteria, these families were classed as belonging to the HNPCC families. Two of them had family members with colorectal cancer, and four with colorectal as well as other HNPCC cancers. The other patients with colorectal cancers were classified as sporadic colorectal cancers as they did not satisfy the Amsterdam criteria.

During the analysis of age and sex of the 7 patients, it was found that three belong to the 31–40 age group, and the remaining four to the 41–50 group. There were six male and one female patient.

In this study we had 6 HNPCC families, and among the examined patients two of them were related (brother and sister). Therefore, 15% of the patients belong to HNPCC families and the rest to the sporadic colorectal cancers.

Loss of heterozygosity (LOH) in APC gene is present in 84.2% of patients, whereas, LOH in DCC1 and DCC2 is present in 42.8% of the patients. Homozygosity in DCC1 was found to be present in 42.8% of the patients (Table 1).

Loss of heterozygosity also referred to as allele imbalance (AI) and homozygosity (H) are indicated.

Two out of seven patients showed microsatellite instability on 4 markers (MSI-H), 2 on 3 and 3 on 2 markers (MSI-H). MSI on BAT40 is present in 84%, on DS123 in 71.4%, on TP53 in 57.1%, on BAT25 and BAT26 in 42.8% of patients (Table 2).

Microsatellite instability is shown in figure 1.

Cancers were diagnosed colonoscopically in the rectum region in three patients, in the ascending colon region in two patients, hepatic flexure in one patient and colon sigmoideum in one patient.

Adenocarcinoma was diagnosed pathohistologically in 5 patients, and adenocarcinoma mucinosum in 2 patients. According to the Astler-Coller classification of colorectal cancer, three of the patients are classified as C1 and four as C2.

Table 1: Genetic changes in tumor suppressor genes

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>Genetic changes</th>
<th>Tumor suppressor genes</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>NM23</td>
</tr>
<tr>
<td>7</td>
<td>AI</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>H</td>
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Table 2: Microsatellite instability in patients with HNPCC syndrome

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>Microsatellite instability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BAT25</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
</tr>
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Discussion

In Bosnia and Herzegovina there has been no study regarding the hereditary forms of colorectal cancer nor is there a national cancer registry. In the Tuzla County in the year 2000, there were a total of 152 patients with colorectal cancer. The incidence rate for men is 6.64 per 100,000 inhabitants and for women 7.42 per 100,000 inhabitants.

In our study, HNPCC was diagnosed in 15% of the patients with colorectal cancer. It is necessary to mention that this research did not cover all patients with the diagnosed colorectal cancer in 2000.

Ponz et al. (10) diagnosed 47 HNPCC-s in 28 families from 1831 cases of colorectal cancers, which was 16% of the total number of patients. Aoltonen et al. (11) gave different results. From 509 tested patients with colorectal cancer only 2% belonged to HNPCC.

We compared our results with both groups of researchers and found that, beside the group of HNPCC they also had a group of ones with suspected HNPCC. That was the group of older patients who meet the Amsterdam criteria. It has to be mentioned, the number of the tested patients in all comparative studies was different.

In our study the patients with HNPCC (N=7), 6 of them (85.7%) had a presence of loss of heterozygosity (LOH) for APC gene, 3 patients (42.8 %) for DCC1 and DCC2, 2 patients (28.4%) for NM23 and 1 patient (14.2%), for p 53 and RB1. These results indicate that there is LOH of tumor suppressor genes for the colorectal cancer located on chromosomes: 5, 13, 17 and 18.

Sasaki et al. (12) researched samples of 22 HNPCC patients. LOH was found on chromosome 5 in 24% cases, on chromosome 14 in 30% cases, on chromosome 17 in 27% cases, on chromosome 18 in 20% and on chromosome 22 in 19% cases. These results indicate that the tumor suppressor genes for colorectal cancer are located on chromosomes 5, 14, 17, 18 and 22.
The analysis of our results show that all 7 patients with HNPCC are highly microsatellite unstable. All patients have microsatellite instability in the area of chromosomes 2p, where the gene hMSH-2 is located, one of mismatch repair genes which are considered as the one with the key role in HNPCC. MSI on BAT 40 was present in 84% in our study and that is characteristic of our sample. So far, DNA variants of BAT 25 have been reported in African Americans with relatively high frequencies and very low in the Japanese population (13).

Ponz et al. (10) tested 18 HNPCC families, and 18 families suspected for HNPCC. Eleven of 18 HNPCC patients showed microsatellite instability (61%) while in the patients suspected for HNPCC only 4 were microsatellite unstable (22%). Three germ line mutations were found in 18 HNPCC families.

Aoltonen et al. (11) prospectively tested colorectal cancer in 509 patients and MSI was present in 63 patterns, what means 12% of patients. The samples of normal tissue in 10 patients had germ line mutation of hMLH1 and hMSH2 genes.

Henry Lynch et al. (14) came to the conclusion, that so far there is no clear gene type marker for HNPCC and it is necessary to continue with research. But, beside the existing mutation of mismatch repair genes, which is recessive, there must be one more somatic mutation. That is the reason why we have to do testing on MSI and on tumor suppressor genes.

In our study, from 7 patients with diagnosed HNPCC in the area of the recto sigmoid region, 57.1% of cancers have been located, while in the area of proximal part of colon 42.9% of cancers were located. Analysis of the distribution of cancers, according to Astler-Coller classification, showed that the level C-1 is represented in 42.8% and the level C2 in 71.4% of cases. The presence of metastatic cancer is significant (p<0.005). Adenocarcinoma was diagnosed in 71.5% cases, while the Adenocarcinoma mucinum is presented in 28.5% cases.

Lynch et al. (14) mention that 65-88% of colorectal cancers within the group of HNPCC, occur in the proximal part of the colon. It is significant that there is a greater presence of mucinous cancer within the framework of HNPCC, and it is diagnosed in the lower level of spreading according to Astler–Coller classification. Those patients have a high possibility of developing metachronous cancers, and 18% of such patients at the first diagnoses of cancer have multiple synchronous tumor.

Why is so difficult to identify patients with Lynch syndrome? There are many challenges, mostly technical, that limit the ability to make the diagnosis. The most comprehensive approach would be to perform genetic testing on every patient with colorectal cancer. However such a strategy would be prohibitively expensive and would not identify all cases (15).

This study indicates the importance of usage of Amsterdam’s and Bethesda criteria and diagnoses of HNPCC as well as registration of HNPCC families. We understood that if we do not make it now, all colorectal cancers are going to be classified and treated as sporadic. We think that the low rate of incidence of colorectal cancer in our region is not realistic but is a result of the absence of screening programs and a registry of cancer.

It is necessary to form a national registry for colorectal cancer as well as registries for HNPCC families in Bosnia and Herzegovina. The instrument which was used in the study, makes a contribution to their formation. It is necessary to design a screening program for individuals with average and high risk for development of colorectal cancer and colonoscopy should be the screening procedure of choice. It is necessary to do genetic screening of HNPCC families as well as genetic consultation. We have to do
direct sequencing to identify mutations. By diagnosing HNPCC it was possible to do a review of surgical treatment, to test and follow their relatives as the persons with high risk for development of colorectal cancer.

**Conclusion**

Despite progress in diagnosis, surgery, chemotherapy and radiotherapy, colorectal cancer presents a real challenge to modern medicine due to its high incidence and mortality rates, as well as a number of etiological unknowns.

HNPCC was diagnosed using The Amsterdam and Bethesda criteria in the group of sporadic colorectal cancer in 15% of cases, and exhibited an MSI-H for the chromosome 2p where the hMSH2 mismatch repair gene is localized.

With our research we obtained data which have a local and global significance. The research should be continued and expanded to a larger number of tested persons to make it possible to compare our results with the results of other authors.

**References**

1. Doto GA. More than a break to the cell cycle. Biochemica et Biophysica Acta, 2000; 1471, 43-56.