Application of RFLP-PCR method for molecular diagnostics of hereditary non-polyposis colorectal cancer (HNPCC)

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INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of cancer deaths, constituting a major public health concern. Epidemiologic studies have revealed a number of risk factors for colorectal cancer including age, family history of colon cancer or inflammatory bowel disease, smoking, alcohol consumption, obesity, and diet. Mutations in MSH2 and MLH1 genes are associated with colon cancer in many studies published to date. The aim of the presented study was to assess the associations of MSH2 and MLH1 genes mutations with colorectal cancer in a Polish population, using the PCR-RFLP method. Mutations in the exon 1 of both genes were detected using the PCR-RFLP method in colorectal patients and healthy individuals. There were no statistical differences in the presence of mutations between colorectal cancer and healthy groups. The PCR-RFLP method is not suitable for the detection of mutant alleles present in less than 5-10% of wild-type alleles. This is probably the reason why in the present study, the analysis did not allow the finding of genetic differences in the first exons of MSH2 and MLH1 genes between healthy individuals and those with the colorectal cancer. It is reasonable to continue studies based on RFLP-PCR, because the costs of this method are low compared to the sequencing method.

Hereditary non-polyposis colorectal cancer (HNPCC) is an autosomal, dominantly inherited tumor, and is associated with germline mutations in mismatch repair (MMR) of genes such as MLH1 and MSH2. Carriers of these mutations are at high risk of colorectal and uterine cancers [6,7]. According to information from the Web of the International Society for Gastrointestinal Hereditary Tumors (InSiGHT), currently, more than 450 different pathogenic mutations have been described in these genes accounting for approximately 750 HNPCC kindreds worldwide [8].

The list of detected mutations in MSH2 and MLH1 genes associated with colorectal cancer is still expanding. Papp et al. in Hungarian HNPCC and suspected-HNPCC families revealed germline mutations in 50% of cases (9 mutations in MLH1 and 9 in MSH2) [9]. Nine of these mutations were newly-detected and not described previously in literature and the InSiGHT mutation database. The majority of reported MLH1 and MSH2 mutations are nonsense, missense, or frameshift mutations, as well as the changes affecting splice sites. However, recent studies have revealed that in some populations the genomic rearrangements are mostly single or multi-exonic deletions or duplications inactivating MLH1 and/or MSH2 [10].

Mutations in MLH1 and MSH2 are found in about 90% of families with identified mutations in DNA mismatch repair genes [11,12]. Many studies confirm the relationship between changes in nucleotide sequences of these genes and colorectal tumorigenesis. Choi et al. in their paper analysed the impact of age, gender, and those associations [8, 13-15]. Research by these authors took into account the ages and
OBJECTIVES

The aim of the study was to identify genetic differences in MSH2 and MLH1 genes between a group of patients with colorectal cancer and a group of healthy individuals. An additionally objective of the study was to detect possible new mutations responsible for HNPCC.

MATERIAL AND METHODS

The study involved a group of 100 patients with an histologically-confirmed diagnosis of colon cancer. The results of genetic testing of genomic DNA of blood samples obtained from the patients were compared with a control group. 100 healthy individuals with negative medical history of any neoplastic process formed the control group.

Mean age in the control group was 55, varying from 21 – 89-years-old (men to women ratio: 44% : 56%). In the study group, the mean age was 56, varying from 30 – 82-years-old (females: 33%).

The most common localization of colorectal cancer in the study group was the rectum (62% of cases). The second most frequent localization was sigmoid and ascending colon (each 10%). In 8% of patient, changes were localized in the transverse colon. Histological types were: 27% adenocarcinoma tubulare, 20% tubulare-villosum adenocarcinoma, adenocarcinoma gelatinosum 6%, and 3% papillare adenocarcinoma. Grading of tumors revealed 50% of the G2 stage, 25% of grade G3 and 8% grade G1.

Sixty samples from the 2 groups of patients were used for molecular research. Thirty of them derived from healthy patients (H) and the other 30 samples were taken from colorectal cancer patients (I). The blood samples were stored at -20°C. Extraction of DNA was performed with QIAamp DNA Blood & Tissue Kit (Qiagen).

Molecular analysis of the MSH2 and MSH1 genes was focused on exon 1. The primers used for amplification of these exon fragments were designed by Pimer 3 v. 0.4.0 program upon nucleotide sequences available at the NCBI database (MLH1 – accession: NM_000249, MSH2 – accession: NG_007110). The primers for the exon 1 MSH2 gene fragment amplification were: F [ACCAGGTGAGGAGGTTT] and R [TGACTGGCATCAAGCTGC].

As a result of amplification of the MLH1 fragment gene with ScfI enzyme in the agarose gel, 2 bands of approx. length 180 and 50 bp appeared, whereas after digestion the same fragment of gene with SphI enzyme, 2 bands were observed with 130 and 100 bp, respectively.

RESULTS

As a result of amplification of the MLH1 fragment gene in agarose gel, one fragment with a length of about 210 bp was observed, and in the case of the MSH2 fragment gene, a fragment approximately 230 bp in length was seen.

After RFLP-PCR analysis of the MSH2 fragment gene with ScfI and SphI enzymes in the agarose gel, 2 bands of approx. length 180 and 50 bp appeared, whereas after digestion the same fragment of gene with SphI enzyme, 2 bands were observed with 130 and 100 bp, respectively.
After HhaI digestion, no differences in bands’ pattern (160 and 50 bp) were observed between the groups. Only one colorectal cancer patient was heterozygous for HhaI MLH1 exon1 RFLP.

DISCUSSION

Restriction Fragment Length Polymorphism (RFLP-PCR) is applied for mutations associated with tumorigenesis. This method was successfully applied for determining N-ethyl-N-nitrosourea-induced mutations in codon 12 of c-H-ras (MspI site 1695–1698), and codon 248 of the p53 tumor suppressor gene (MspI site 14067–14070) in human skin fibroblasts [19]. Mutant-type K-ras gene was found in plasma DNA samples in plasma of patients with pancreatic carcinoma [20] or mutations in epidermal growth factor receptor (EGFR), which are strong determinants of tumor response to EGFR tyrosine kinase inhibitors in non-small lung cell cancers (NSCLCs) [21].

In the present study, there were no genetic differences between both groups of patients – healthy individuals and patients with CRC. In only one case (locus MLH1), a CRC patient was heterozygote for MLH1 HhaI. The lack of differences between the control group and colorectal cancer group involved only 1 exon analysis, and is not proof that in the area of both analyzed genes there are no mutations correlated with the appearance of Hereditary non-polyposis colorectal cancer in people.

Both genes, MSH2 and MLH1, have a very complex construction. MSH2 is composed of 16 exons, and only the encoded part of mRNA is 3,166 nucleotides in length (NCBI accession: NG_007110). The encoded part of mRNA is constructed of 2,935 nucleotides (NCBI accession: NG_007110). Mutant-type K-ras gene was found in plasma DNA samples in plasma of patients with pancreatic carcinoma [20] or mutations in epidermal growth factor receptor (EGFR), which are strong determinants of tumor response to EGFR tyrosine kinase inhibitors in non-small lung cell cancers (NSCLCs) [21].

In the subsequent stage of researches, the sequencing of both fragments of genes will be carried out. Perhaps this analysis will allow the finding of any mutations in another part of the nucleotide sequence in exon one of MSH2 and MLH1 RFLP fragment genes connected with HNPCC.

PCR-RFLP is widely used and one of the simplest methods for detecting mutations in cancer-related genes, and for genotyping a wide range of other human diseases [21-26]. Halilasos et al. claim that a drawback for the application of this method in the field of cancer, is that it cannot detect mutant alleles present in less than about 5–10% of wild-type alleles [27]. This could be an explanation why in present study RFLP-PCR analysis did not detect genetic differences in the first exons of MSH2 and MLH1 genes between healthy individuals and patients with hereditary non-polyposis colorectal cancer. However, it is worth emphasizing that RFLP-PCR is only a screening method, and it would be impossible to find a mutation in sequences recognized by the 4 restriction enzymes (Ddel, HhaI, Sfil and Sphtl). Nevertheless, there is a need for continuing research based on RFLP-PCR because of the advantages of this method of low costs for the analysis, compared to sequencing. It is possible that in other fragments of the MHS2 and MLH1 genes the method will allow the detection of mutations presented only in patients with cancer, without substantially increasing the effort and cost associated with molecular investigations.

CONCLUSIONS

1. PCR-RFLP analysis of exon 1 MLH1 gene with HhaI enzyme revealed the presence of mutation in one case of colorectal cancer.
2. RFLP-PCR method using HhaI, Ddel restrictases for exon 1 MLH1 gene and Sphtl for exon 1 MHS2 genes is not capable of detecting any relevant mutations.

REFERENCES


