Journal of Pre-Clinical and Clinical Research, 2011, Vol 5, No 2, 70-73
www.jpccr.eu

ORIGINAL ARTICLE

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

Andrzej Prystupa[1], Magdalena Buś-Kicman[2], Grzegorz Dzida[1], Roman Styliński[3], Paweł Piwowarczyk[4], Małgorzata Sawa[1], Małgorzata Janowska[3], Jerzy Mosiewicz[1]

[1] *Department of Internal Medicine, Medical University, Lublin, Poland*
[2] *Department of Biological Bases of Animal Production, University of Life Sciences, Lublin, Poland*
[3] *Department of General Surgery with Endoscopy Unit, Medical University, Lublin, Poland*
[4] *Department of Anesthesiology and Intensive Care Unit, Medical University, Lublin, Poland*

Abstract

Colorectal cancer 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 MLH2 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.

Keywords

colorectal cancer, RFLP, hereditary nonpolyposis

INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of cancer deaths. More than 550,000 Americans die each year of colon or rectal cancer, constituting a major public health concern [1]. Annually, approximately 11,000 new cases of CRC are diagnosed in Poland, while the number of deaths caused by CRC approaches 8,000. Five-year survival does not exceed 20%. The majority of colorectal cancers originate from adenomas. The risk of malignant transformation of a benign lesion is approximately 2% annually [2].

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. According to the CDC (Center for Disease Control and Prevention), those who have a family history of colorectal cancer are at higher risk for developing colorectal cancer themselves [2].

Predisposing factors for colorectal carcinoma in children and young adults include hereditary conditions affecting the bowel (polyposis and non-polyposis syndromes), inflammatory bowel disease, and radiation exposure. Approximately 15-20% of colorectal cancer patients have familial colon cancer without a defined genetic pattern [3], about 5% have hereditary non-polyposis colon cancer [4], and 1% have hereditary polyposis syndromes [5].

Corresponding author: Andrzej Prystupa, Department of Internal Medicine, Medical University, Staszica 16, 20-081 Lublin, Poland.
E-mail: aprystup@mp.pl

Received: 10 September 2011; accepted: 21 December 2011

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

gender of patients. The risks of developing cancer by the age of 70 were 60%, and 47% among the male and female carriers of any MMR mutation, respectively. Additionally, among *MLH1* mutation carriers, males had significantly higher risks than females at all ages, while the risks were similar in *MSH2* carriers. The relative risk associated with *MLH1* was almost constant with age, while for *MSH2* decreased with age [14]. On the other hand, in a population-based study, *MLH1* and *MSH2* mutation carriers identified a mean age at diagnosis closer to 54 years for men and 60 years for women [16].

The results of many studies have strong, high correlations between mutations in MSH2/MLH1 and occurrence of HNPCC. Additionally, new mutations are still being detected. The aim of the presented study was exploration of the first exon of MSH2 and MLH1 genes to detect genetic differences between patients with and without histologically-confirmed CRC.

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 formulations 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 [GCCCCATGTACTTGATCACC], and for exon 1 the fragment of MLH1 gene primers were: F [TGACTGGCATTCAAGCTGTC] and R [TTCACCACTGTCTCGTCCAG].

The amplification product of MSH2 gene fragment was 229 bp, and for MLH1 fragment gene- 214 bp. The PCR conditions for both amplifications were performed in 30 µl reaction volumes containing, on average, 20 ng/µl of DNA, 3 µl of 10 × PCR buffer, 6 µl of 1 × Q solution (Qiagen), 2.5 mM $MgCl_2$, 200 µM each of dNTP, 0.2 µM each of primers, and 0.5 U of Taq polymerase (Qiagen). The following PCR conditions profile was used for amplifying both fragments: initial denaturation for 3 min. at 94°C, followed by 35 cycles: 1 min. at 94°C; 45 s at 58°C; 45 s at 72°C; final extinction for 10 min. at 72°C.

The choice of restriction enzymes was made on NEBcutter V2.0 software [18], *Dde*I and *Hha*I for the MLH1 gene, and *Sfc*I, *Sph*I of the MSH2 gene were selected reaction conditions with corresponding enzymes. The restriction analysis of amplified fragments of both genes was conducted according with the manufacturer's instructions. Electrophoresis of the amplified genes fragments was performed in 3.5% agarose gel with DNA ladders: GeneRuler 50bp DNA Ladder ready-to-use and GeneRuler 100bp DNA Ladder Plus ready-to-use. Analysis was performed for restriction fragment detection with a computer coupled a CCD camera using Software.

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 *Scf*I enzyme in the agarose gel, 2 bands of approx. length 180 and 50 bp appeared, whereas after digestion the same fragment of gene with *Sph*I enzyme, 2 bands were observed with 130 and 100 bp, respectively.

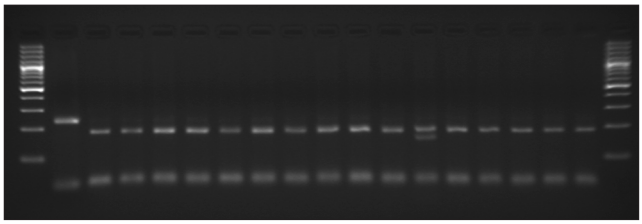

Figure 1. Fragment of MSH2 gene digested with ScfI enzyme.

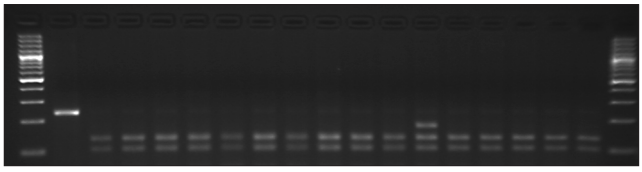

Figure 2. Fragment of MSH2 gene digested with SphI enzyme.

In restriction analysis of the MLH1 fragment gene with using *Dde*I enzyme, 2 bands were observed - 120 i 90 bp, respectively.

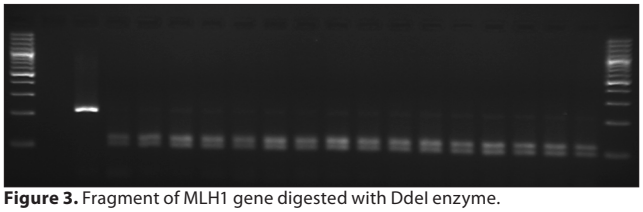

Figure 3. Fragment of MLH1 gene digested with DdeI enzyme.

After *Hha*I digestion, no differences in bands' pattern (160 and 50 bp) were observed between the groups. Only one colorectal cancer patient was heterozygous for *Hha*I 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-*rasl* (*Msp*I site 1695–1698), and codon 248 of the p53 tumor suppressor gene (*Msp*I 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). *MLH1* contains 22 exons, and the encoded part of mRNA is constructed of 2,935 nucleotides (NCBI accession: NG_007109). Due to the long sequences of both genes, the use of screening methods to discover genetic differences between people with and without diagnosed cancer is well-founded. Unfortunately, not every time applying of RFLP-PCR could indicate mutations in particular part of chosen sequences because the restriction enzymes allows the detection of mutation only on the part of its restriction side.

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* fragment genes connected with HNPCC.

PCR-RFLP is widely used and one of the simplest method for detecting mutations in cancer-related genes, and for genotyping a wide range of other human diseases [21-26]. Haliassos 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. Hoever, 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 (*Dde*I, *Hha*I, *Sfc*I and *Sph*I). 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 *Hha*I, *Dde*I restrictases for exon 1 MLH1 gene and *Sph*I for exon 1 MHS2 genes is not capable of detecting any relevant mutations.

REFERENCES

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ. Cancer statistics, 2008. CA Cancer J Clin 2008;58(2):71-96.
2. Colorectal Cancer Risk Factors. http://www.cdc.gov/cancer/colorectal/basic_info/risk_factors.htm (access:2011.08.19)
3. Boutron MC, Faivre J, Quipourt V, Senesse P, Michiels C. Family history of colorectal tumours and implications for the adenomacarcinoma sequence: a case control study. Gut 1995;37:830-4.
4. Marra G, Boland CR. Hereditary non-polyposis colorectal cancer: the syndrome, the genes, and historical perspectives. J Natl Cancer Inst 1995;87(15):1114-25.
5. Vogelstein B. Genetic testings for cancer: the surgeon's critical role. Familial colon cancer. J Am Coll Surg 1999;188(1):74-9.
6. Kładny J, Lubiński J. Dziedziczny niepolipowaty rak jelita grubego [Hereditary non-polyposis colorectal cancer]. Pol Przegl Chir 1996;68:728-734.
7. Lynch HT, de la Chapelle A. Genetic susceptibility to non-polyposis colorectal cancer. J Med Genet 1999;36(11):801-18.
8. Peltomaki P, Vasen H. Mutations associated with HNPCC predisposition-Update of ICG-HNPCC/INSiGHT mutation database. Dis Markers 2004;20:269-276.
9. Papp J., Kovacs M. E., Olah E. Germline MLH1 and MSH2 mutational spectrum including frequent large genomic aberrations in Hungarian hereditary non-polyposis colorectal cancer families: Implications for genetic testing. World J Gastroenterol 2007;13(19): 2727-2732.
10. Taylor CF, Charlton RS, Burn J, Sheridan E, Taylor GR. Genomic deletions in MSH2 or MLH1 are a frequent cause of hereditary non-polyposis colorectal cancer: identification of novel and recurrent deletions by MLPA. Hum Mutat 2003;22(6):428-433.
11. Lynch HT, de la Chapelle A. Hereditary colorectal cancer. N Engl J Med 2003; 48:919-932.
12. Baudhuin LM, Burgart LJ, Leontovich O, Thibodeau SN. Use of microsatellite instability and immunohistochemistry testing for the identification of individuals at risk for Lynch syndrome. Fam Cancer 2005;4:255-265.
13. Charbonnier F, Raux G, Wang Q, Drouot N, Cordier F, Limacher J-M. Saurin J-C, Puisieux A, Olschwang S, Frebourg T. Detection of exon deletions and duplications of the mismatch repair genes in hereditary nonpolyposis colorectal cancer families using multiplex polymerase chain reaction of short fluorescent fragments. Cancer Res 2000;60(11):2760-2763.
14. Choi Y-H, Cotterchio M, McKeown-Eyssen G, Neerav M, Bapat B, Boyd K, Gallinger S, McLaughlin J, Aronson M, Briollais L. Penetrance of colorectal cancer among MLH1/MSH2 carriers participating in the colorectal cancer familial registry in Ontario. Hered Cancer Clin Pract 2009;7(1):14.

15. Gargiulo S, Torrini M, Ollila S, Nasti S, Pastorino L, Cusano R, Bonelli L, Battistuzzi L, Mastracci L, Bruno W, Savarino V, Sciallero S, Borgonovo G, Nyström M, Bianchi-Scarra`G, Mareni C, Ghiorzo P. Germline MLH1 and MSH2 mutations in Italian pancreatic cancer patients with suspected Lynch syndrome. Fam Cancer 2009; 8(4):547-553.
16. Hampel H, Stephens JA, Pukkala E, Sankila R, Aaltonen LA, de la Chapelle A. Cancer risk in hereditary nonpolyposis colorectal cancer syndrome. Gastroenterology 2005;129(2):415-421.
17. http://frodo.wi.mit.edu/primer3/ (access: 2011.08.19)
18. Vincze T, Posfai J, Roberts RJ. NEBcutter: a program to cleave DNA with restriction enzymes. Nucleic Acids Res 2003;31(13):3688-3691.
19. Pourzand C, Cerutti P. Genotypic mutation analysis by RFLP/PCR. Mutat Res 1993;288(1):113-21.
20. Castells A, Puig P, Móra J, Boadas J, Boix L, Urgell E, Solé M, Capellà G, Lluís F, Fernández-Cruz L, Navarro S, Farré A. K-ras mutations in DNA extracted from the plasma of patients with pancreatic carcinoma: diagnostic utility and prognostic significance. J Clin Oncol 1999;17(2):578-84.
21. Eiken H G, Odland E, Boman H, Skjelkvale L, Engebretsen L F, Apold J. Application of natural and amplification created restriction sites for the diagnosis of PKU mutations. Nucleic Acids Res 1991;19(7):1427-1430.
22. Bazrafshani MR, Ollier WE, Hajeer AH. A novel PCR-RFLP assay for the detection of the single nucleotide polymorphism at position-1082 in the human IL-10 gene promoter. Eur J Immunogenet 2000;27(3):119-120.
23. Friedman KJ, Highsmith WE Jr, Prior TW, Perry TR, Silverman LM. Cystic fibrosis deletion mutation detected by PCR-mediated site-directed mutagenesis. Clin Chem 1990;36(4):695-696.
24. Parsons BL, Heflich RH. Genotypic selection methods for the direct analysis of point mutations. Mutat Res 1997;387(2):97-121.
25. Plendl H, Siebert R, Steinemann D, Grote W. High frequency of the N34S mutation in the SPINK1 gene in chronic pancreatitis detected by a new PCR-RFLP assay. Am J Med Genet 2001;100(3):252-253.
26. Medina-Arana V, Barrios Y, Fernández-Peralta A, Herrera M, Chinea N, Lorenzo N, Jiménez A, Martín-López JV, González-Hermoso F, Salido E, González-Aguilera JJ. New founding mutation in MSH2 associated with hereditary nonpolyposis colorectal cancer syndrome on the Island of Tenerife. Cancer Lett 2006;244(2):268-273.
27. Haliassos A, Chomel JC, Grandjouan S, Kruh J, Kaplan JC, Kitzis A. Detection of minority point mutations by modified PCR technique: a new approach for a sensitive diagnosis of tumor-progression markers. Nucleic Acids Res 1989;17(20):8093-8099.