Association of *H-ras* polymorphisms and susceptibility to sporadic colon cancer

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Abstract. High incidence of colon cancer worldwide indicates the importance of studying genetic alterations that lead to its carcinogenesis. Two polymorphisms in *H-ras* gene, hexanucleotide tandem repeats in the first intron and SNP 81T>C in the first exon, that might be connected with susceptibility to cancer have been described. The aim of our study was to investigate these loci in Croatian population and to determine if any of them is connected with susceptibility to colon cancer in our population. Two hundred healthy volunteers and 200 colon cancer patients were genotyped using PCR and RFLP methods. We noted statistically significant difference in genotype distribution at hexanucleotide locus between healthy population and colon cancer patients (p=0.013) but not in allelic distribution. At SNP 81 T>C statistically significant difference in distribution of both genotypes ($p=9.15x10^{-6}$) and alleles ($p=2.77x10^{-6}$) was found. No differences were found between genders.

Introduction

Colorectal cancer is one of the most frequent types of cancer worldwide and is on the third place of mortality of cancer (1). One of the most important gene families involved in colorectal carcinogenesis is the Ras family of genes. It is a family of proto-oncogenes in which numerous mutations have been recognized and associated with the risk of various cancers (2,3).

The majority of proto-oncogenes are involved in cell signaling (4) and therefore in the cell growth and proliferation. Mutation in this type of genes can cause abnormal growth which is a predisposition for cancer development. Ras gene family consists of three genes: *H-ras, K-ras* and *N-ras*. They all encode for 21 kDa proteins that bind to the inner surface of the cell membrane. These proteins participate in regulation of the cell differentiation, development and proliferation with their GTPase activity (2,3,5,6). These three genes as well as their protein products are structurally and functionally very similar (2,3,6). They are all expressed in most normal tissues but at different levels (7). It has also been observed that different *ras* genes are activated in different human tumours (2,3).

Harvey-*ras* (*H-ras*) gene is located on chromosome 11 (8). It was the first *ras* gene that was identified in its altered state in the EJ bladder carcinoma cell line (9). Since then a number of mutations were described inside this gene that are implicated in the development of various type of cancer. *H-ras* gene can participate in carcinogenesis in two ways: first if the protein function is modified and second if its expression is enhanced (2,3,10).

Taparowsky *et al* (11) described a point mutation that is thought to be involved in generating cancer, a single nucleotide polymorphism in codon 27 in the first exon (SNP 81T>C, rs12628) of *H-ras* gene. Studies were performed to explore the possible influence of SNP 81T>C on susceptibility to various types of cancer (12-16) and syndromes (17). Some authors consider it as a low penetrance gene predisposition factor for gastrointestinal cancer (16).

Tandemly repeated hexanucleotide locus is located 83 bp upstream of the 5' end of exon 1 (18). Two alleles P1 and P2 were described. P1 has four repeats of a hexanucleotide sequence GGGCCT and P2 has two of the repeats. Tanci *et al* (19) found the third allele P3 based on its electrophoretic mobility that has three hexanucleotide repeats. P2 and P3 alleles possess perfect repeats while P1 possesses three perfect and one imperfect repeats.

We focused our research on these two described polymorphic loci due to contradictory results from previous studies that have not yet resolved their contribution to tumourigenesis. The aim of this study was to determine the allele frequencies of this two polymorphisms in Croatian population. To investigate whether any of them contributes to predisposition to colon cancer the control group results were compared with those of colon cancer patients.

Patients and methods

Patients. We analyzed samples from 200 patients with sporadic colon cancer and 200 unrelated healthy volunteers. All specimens used in our study were obtained from the

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Croatian Tumour and DNA Bank for basic research, Ruder Boskovic Institute, Zagreb, Croatia (20). Relevant information on all subjects (demographic characteristics, age, sex and family history of cancer) were noted. Sex distribution among controls was 104 female versus 96 male and among patients 94 female versus 106 male. Mean age of healthy population was 66.1 and that of colon cancer patients 64.9. All colon adenocarcinoma patients had no family history of hereditary cancer. Diagnosis was based on standard diagnostic procedures and confirmed histopathologically. Samples of resected colon tissue were snap-frozen in liquid nitrogen and stored at -80˚C until further use. DNAs were extracted from histologically normal colon mucous adjacent (>15 cm from the tumour) to adenocarcinoma.

Control subjects were randomly selected among unrelated volunteers. Exclusion criteria were malignant disease in family history. Their DNAs were extracted from blood samples. This study was approved by the Ethics Committee and all the participants gave their written informed consent.

DNA isolation. Genomic DNAs were isolated from tumour tissue samples taken from patients and from peripheral blood taken from healthy controls using proteinase K digestion and phenol chloroform extraction.

Hexanucleotide polymorphism analysis. DNA was used as a template in an amplification reaction with specific pair of primers: forward 5'-CTG TGG GTT TGC CCT TCA GA-3' and reverse 5'-CTC CTA CAG GGT CTC CTG CC-3'. Total reaction volume was 25 μl containing 200 ng of genomic DNA, 10 pmol of each primer, 50 μM of each dNTP and 1 U Taq DNA polymerase (Applied Biosystems). PCR reactions were carried out in GeneAmp PCR System 2400 (Applied Biosystems) for 35 cycles with 30 sec at 96˚C, 30 sec at 64˚C and 30 sec (adding 1 sec for each following cycle) at 72˚C. PCR products were analyzed on Spreadex® EL 300 Wide Mini gel (Elchrom Scientific). Five microliters of each sample mixed with loading buffer was loaded onto Spreadex® gel. Electrophoresis was performed at 10 V/cm at 55˚C for 3 h. SYBR Gold was used for staining the gel.

Single nucleotide polymorphism analysis. DNA was amplified using specific pair of primers: forward 5'-CTTGGCAGGT GGGGCAGGAGA-3' and reverse 5'-GGCACCTGGACGG CGGCGCTAG-3'. Total reaction volume was 25 μl containing 200 ng of genomic DNA, 10 pmol of each primer, 50 μM of each dNTP and 1U Taq DNA polymerase (Applied Biosystems). PCR reactions were carried out in GeneAmp PCR System 2400 (Applied Biosystems) for 35 cycles with 30 sec at 94˚C, 10 sec at 60˚C and 45 sec (adding 1 sec for each following cycle) at 72˚C.

Five microliters of each PCR product was digested overnight at 37˚C with 3 U of *Dra*II (New England Biolabs) restriction enzyme in a total reaction volume of 25 μl. RFLP samples were analyzed by vertical polyacrylamide electrophoresis. Fourteen microliters of each sample mixed with loading buffer was loaded onto 8% non-denaturing polyacrylamide gel. It was performed in 1X TBE (Tris/borate/EDTA) buffer for 2 h at 40 W at room temperature. The gel was silver stained.

Table I. Hexanucleotide polymorphism in healthy volunteers and colon cancer patients.

Hexanucleotide repeats	Healthy controls n=200 (%)	Colon cancer patients n=200 (%)	p-value
Genotypes			
P1/P1	89 (44.5)	99 (49.5)	0.013
P1/P2	45 (22.5)	51 (25.5)	
P2/P2	11 (5.5)	7 (3.5)	
P2/P3	14 (7.0)	5 (2.5)	
P3/P3	2 (1.0)	11 (5.5)	
P1/P3	39 (19.5)	27 (13.5)	
Alleles			
P1	262 (65.5)	276 (69.0)	0.536
P2	81 (20.3)	70 (17.5)	
P3	57 (14.2)	54 (13.5)	

Table II. SNP 81T>C polymorphism in healthy volunteers and colon cancer patients.

SNP 81T>C	Healthy controls n=200 (%)	Colon cancer patients n=200 (%)	p-value
Genotypes			
TT	85 (42.5)	121 (60.5)	9.15×10^{-6}
TC	85 (42.5)	73 (36.5)	
CC	30 (15.0)	6 (3.0)	
Alleles			
T	255 (63.8)	315 (78.8)	2.77×10^{-6}
C	145 (36.2)	85 (21.2)	

Results

In this study two *H-ras* polymorphisms were studied. Genotype frequencies for both hexanucleotide polymorphism and SNP 81T>C were distributed in accordance with the Hardy-Weinberg equilibrium. Genotyping results are summarized in Tables I and II.

To analyze hexanucleotide polymorphism we used PCR amplification with specific primers. Fig. 1 illustrates a Spreadex gel with all three alleles represented P1 (118 bp), P2 (106 bp) and P3 (112 bp). Genotype distribution and allele frequencies of hexanucleotide locus in patients and controls are presented in Table I. Genotype frequencies were 44.5, 22.5, 5.5, 7, 1 and 19.5% in healthy population, and 49.5, 25.5, 3.5, 2.5, 5.5 and 13.5% in colon cancer for P1/P1, P1/P2, P2/P2, P2/P3,

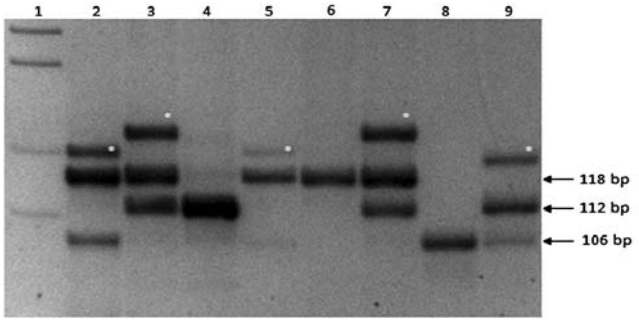

Figure 1. Hexanucleotide polymorphism analysis by submerged gel electrophoresis on Spreadex EL 300 gel (3 h, 10 V/cm, 55˚C). Lane 1, DNA marker pBR322 DNA *Msp*I digest; lanes 2 and 5, heterozygotes P1/P2; lanes 3 and 7, heterozygotes P1/P3; lane 4, homo-zygote P3/P3; lane 6, homozygote P1/P1; lane 8, homozygote P2/P2; lane 9, heterozygote P2/P3. Allele P1, 118 bp; allele P2, 106 bp; allele P3, 112 bp; *heteroduplex.

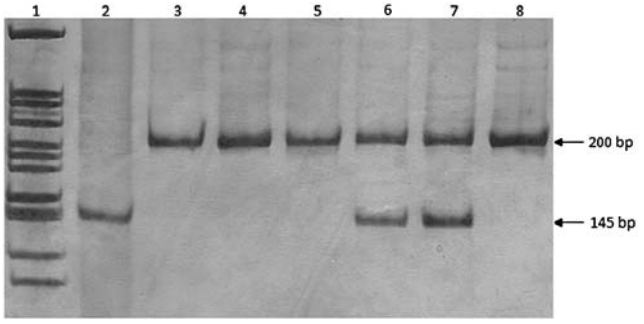

Figure 2. SNP 81T>C polymorphism analysis using PCR-*Dra*III RFLP method on 8% non-denaturing polyacrylamide gel. Lane 1, DNA marker pBR322 DNA *Msp*I digest; lane 2, homozygote CC; lanes 3, 4, 5 and 8, homozygotes TT; lanes 6 and 7, heterozygotes TC. Allele T, 200 bp; allele C, 145+55 bp.

P3/P3 and P1/P3 genotype, respectively. Allele frequencies were 65.5, 20.3 and 14.2% in healthy population and 69, 17.5 and 13.5% in colon cancer for P1, P2 and P3, respectively. There were significant differences between patients and controls genotype distribution. Genotype P3/P3 was more common in colon cancer patients and P2/P2 and P2/P3 genotypes in healthy population. There were no significant differences in allelic distribution.

To analyze SNP 81T>C we used PCR-DraIII RFLP method. Fig. 2 illustrates *Dra*III RFLP analysis of SNP 81T>C polymorphism. The PCR products were digested with *Dra*III to detect alleles T (200 bp) and C (145+55 bp). Genotype distribution and allele frequencies of SNP 81T>C polymorphism in patients and controls are presented in Table II. Genotype frequencies were 42.5, 42.5 and 15% in healthy population and 60.5, 36.5 and 3% in colon cancer for TT, TC and CC, respectively. Allele frequencies were 63.8% and 36.2% in healthy population and 78.8 and 21.2% in colon cancer for T and C alleles, respectively. Statistically significant difference was observed in both genotype distribution and allelic distribution between healthy controls and colon cancer patients at this polymorphic locus. Allele -81C was more common in healthy volunteers in comparison to colon cancer patients.

Table III. Genotype and allelic distribution at hexanucleotide polymorphic locus between male and female population in healthy volunteers.

Hexanucleotide repeats	Male n=96 (%)	Female n=104 (%)	p-value
Genotypes			
P1/P1	38 (39.6)	51 (49.0)	0.513
P1/P2	25 (26.0)	20 (19.2)	
P2/P2	5 (5.2)	6 (5.8)	
P2/P3	7 (7.3)	7 (6.7)	
P3/P3	2 (2.1)	0 (0.0)	
P1/P3	19 (19.8)	20 (19.2)	
Alleles			
P1	120 (62.5)	142 (68.3)	0.477
P2	42 (21.9)	39 (18.7)	
P3	30 (15.6)	27 (13.0)	

Table IV. Genotype and allelic distribution at hexanucleotide polymorphic locus between male and female population in colon cancer patients.

Hexanucleotide repeats	Male n=106 (%)	Female n=94 (%)	p-value
Genotypes			
P1/P1	56 (52.8)	43 (45.7)	0.137
P1/P2	22 (20.7)	29 (30.8)	
P2/P2	6 (5.7)	1 (1.1)	
P2/P3	1 (0.9)	4 (4.3)	
P3/P3	5 (4.7)	6 (6.4)	
P1/P3	16 (15.1)	11 (11.7)	
Alleles			
P1	150 (70.8)	126 (67.0)	0.723
P2	35 (16.5)	35 (18.6)	
P3	27 (12.7)	27 (14.4)	

Comparison of genotype distribution and allelic distribution at hexanucleotide locus between genders is summarized in Table III for healthy population and Table IV for population of colon cancer patients. No differences were observed between male and female population.

Distribution of genotypes and alleles at SNP 81T>C between genders is summarized in Tables V and VI. No differences were observed between male and female population.

Table V. Genotype and allelic distribution at SNP 81T>C locus between male and female population in healthy volunteers.

SNP 81T>C	Male n=96 (%)	Female n=104 (%)	p-value
Genotypes			
TT	40 (41.7)	45 (43.3)	0.961
TC	41 (42.7)	44 (42.3)	
CC	15 (15.6)	15 (14.4)	
Alleles			
T	121 (63.0)	134 (64.4)	0.771
C	71 (37.0)	74 (35.6)	

Table VI. Genotype and allelic distribution at SNP 81T>C locus between male and female population in colon cancer patients.

SNP 81T>C	Male n=106 (%)	Female n=94 (%)	p-value
Genotypes			
TT	62 (58.5)	59 (62.8)	0.706
TC	40 (37.7)	33 (35.1)	
CC	4 (3.8)	2 (2.1)	
Alleles			
T	164 (77.4)	151 (80.3)	0.470
C	48 (22.6)	37 (19.7)	

Discussion

H-ras gene is found to be frequently mutated in human tumours (2,3) and its alterations were widely studied. Polymorphic loci of our interest, hexanucleotide repeats and SNP 81T>C, were examined on tissue samples of different types of human tumours - bladder, breast, lung, skin, oral, gastrointestinal (12-16,21-23). In this study we investigated the correlation of these loci with the predisposition to sporadic colon cancer in Croatian population.

High frequency of heterozygosity at hexanucleotide locus was shown, which makes it a suitable system for genetic linkage and loss of heterozygosity studies in tumour cells (19). A number of reports described the linkage disequilibrium of this hexanucleotide polymorphism and the VTR region at the 3'-end of the *ras* gene and its influence on the cell survival (18,21,23).

Our findings on allelic distribution were in accordance with other results. The highest frequency is of P1 allele (65.5%) while P2 (20.3%) and P3 (14.2%) are shown to be much rarer in the healthy population. The same distribution of P1 (69%), P2 (17.5%) and P3 (13.5%) was found in colon cancer patients. Statistically significant difference was noted in distribution of hexanucleotide polymorphism genotypes between healthy volunteers and colon cancer patients (p=0.013). P2/P2 is found in 5.5% and P2/P3 in 7% of control samples and 3.5 and 2.5% patients respectively. As opposed to those genotypes, P3/P3 is found in 5.5% of colon cancer patients and in only 1% of controls.

There were no differences found among genders regarding distribution of genotypes and alleles in either the healthy population or patients. Our results on healthy female population differ from results obtained by Papadakis *et al* (23) mostly regarding the occurence of P1, P2 and P3 homozygosity. They associated increased presence of P1 and P2 homozygosity and absence of P3 homozygosity with increased risk for breast carcinogenesis while we noted no significant difference in P1 homozygosity, increased presence of P3 and decreased presence of P2 homozygosity in colon cancer patients. This different genotype distribution can be due to different tumour origin or to genetic differences among populations.

Silent mutation at position 81 in exon 1 has no effect on p21 structure and function (11,24) and therefore is not significant for carcinogenesis (25). As opposed to this conclusion, it was reported that SNP 81T>C can indicate an increased risk of skin (12), oral (15), bladder (13) and gastric cancer (16). Although it does not alter the amino acid sequence of the protein it may affect the expression of the gene inducing overexpression (26). It is possible that it is linked to another polymorphic locus inside regulatory intronic region (13).

Differences among healthy population of various ethnic groups (Chinese, German, Indian) are observed (16). All authors agree that the frequency of allele T is much higher than of allele C, however the frequency of genotypes differ. Higher frequency of heterozygote TC than of homozygote TT is observed only among controls in Swedish population (14). We observed the same frequency of TC and TT genotypes in the healthy population (42.5%). The frequency of homozygote CC among healthy Croatian individuals is 15% and that is the highest frequency of this genotype described in the present studies.

When comparing healthy population and colon cancer patients, we found statistically significant differences in genotype distribution ($p=9.15x10^{-6}$) and in allelic distribution ($p=2.77x10^{-6}$). The frequency of allele C was statistically significantly lower in the group of colon cancer patients in comparison with healthy population. CC genotype was lower and TT was higher in colon cancer patients in comparison with healthy population and these findings were also statistically significant. Our results are in accordance with the results of Sanyal *et al* (14) who reported decrease of allele C and CC genotype in cancer patients compared to healthy controls. In other studies that reported SNP 81T>C as a potential risk for carcinogenesis, in bladder, oral and gastric cancer, it was affiliated with higher occurence of allele C (13,15,16). This can be due to the different type of cancer or to ethnic differences between populations that were the subject of the studies.

Some authors reported the possibility of tumour suppressor role of *H-ras* gene (27-29). Loss of *H-ras* function could

be necessary for malignant phenotype development (22). Both hexanucleotide locus (10) and SNP 81T>C (13,26) are postulated to be involved in expression of the *H-ras* gene. Higher frequency of allele C in healthy population can be associated with normal expression of this gene in healthy colon cells. The role that *H-ras* gene has in human carcinogenesis is yet to be resolved in future studies on gastrointestinal and other human tumours.

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