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Association of EGF and p53 gene polymorphisms and colorectal cancer risk in the Slovak population

Research Article

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Abstract: During the transformation process single nucleotide polymorphisms (SNPs) of key genes, such as *p53* Arg72Pro or *EGF* A61G, may mediate various cellular processes. These variants may be associated with colorectal cancer risk (CRC), but conflicting findings have been reported. The purpose of this study was to determine the association of the SNPs in 5´UTR of *EGF* A61G and *p53* Arg72Pro and CRC in the Slovak population. The present case-control study was carried out in 173 confirmed CRC patients and 303 healthy subjects. Genotyping was performed by PCR-RFLP methods. Significant association was observed between age and CRC risk (p=0.001). Lower CRC risk was seen in younger patients carrying genotype *p53* Arg72Pro (0.14; 95% CI 0.02-0.99, p=0.049). Gender-stratified analysis showed a significant inverse association of the polymorphism *EGF* G61G with CRC risk (0.48; 95% CI 0.2-0.9, p=0.04) only in male patients. Tumour site genotype distribution revealed that female patients with localized colon cancer were significantly associated with *p53* Pro72Pro genotype (4.0; 95% CI 1.27-12.7, p=0.04) whereas the cancer of rectosigmoid junction was associated with the EGF G61G genotype (4.5; 95% CI 1.2-16.97, p=0.02). Combination of *p53* Arg72Pro or *EGF* A61G polymorphisms were not associated with CRC risk by using logistic regression.

Keywords: *Colorectal cancer • EGF • p53 • Gene polymorphism*

1. Introduction

Sporadic colorectal cancer is a multifactorial disease arising from interaction between genetic background and environmental factors, such as diet or lifestyle; however, the exact role of the genetic background to sporadic CRC remains unclear [1]. In the last decades Europe has seen a widespread growth in the incidence of CRC, especially in Eastern populations. Colorectal cancer was the third major cause of death in Europe in 2008 (13.5% of total death) almost equally distributed between sexes [2]. In the Czech Republic and Slovakia incidence rates among men have not only exceeded the peak incidence observed in the United States and other long-standing developed nations but still continue to increase [3]. Thus, the Central European population could serve as a suitable model population for the study of the genetic background to CRC.

The tumour suppressor protein – *p53* normally inhibits proliferation of cells with DNA damage and regulates various processes that may contribute to its tumour suppressive functions, including glycolysis, autophagy, cell mobility, microRNA processing, ageing and suntanning

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[4]. Changes in the p53 amino acid sequence can alter the ability of p53 to bind response elements of target genes, alter recognition motifs for post-translational modifications, or alter the protein stability and interactions with other proteins [5]. The gene for *p53* is located on the short arm of chromosome 17p13.1. A common SNP, referred to as Arg72Pro, is located in the proline-rich region in exon 4 in the segment of *p53* [5] and encodes either an arginine amino acid (CGC, Arg72) or a proline residue (CCC, Pro72). This region is required for the growth suppression and apoptosis mediated by p53 but not for cell cycle arrest [6]. Arg72 form of p53 is a more efficient inducer of apoptosis than Pro72, and thus may increase the responsiveness to chemotherapy [7-9]. Pro72 is more efficient in transactivating p21 and inducing cell cycle arrest [10]. The earlier studies have reported a controversal results about the association between Pro72Pro mutant phenotype and the CRC [6,11-18]. These discrepancies have been suggested to be due to the race-specific effects, as the Pro has been ancestral allele (~ 95% allele frequence in Africans), and the frequency of the Arg allele progressively increased as populations migrated further North [19-20].

The other molecular players such as growth factors play an important role in the development of the CRC. In this paper we focus on one of them, the epidermal growth factor (*EGF*) gene, located at 4q25-27. It encodes a ligand EGF for the EGFR, that is known to homodimerize, then to transphosphorylate several tyrosine kinase domains and activates a series of intracellular signaling networks including PI3K/AKT, Ras/Erk, JAK/STAT [21-25]. These networks activate or deactivate some transcription factors regulating some proteins responsible for the death or survival of cell. Expression of both EGF and EGFR have been described to be significantly increased in a various human tumours including breast [26], lung [27-28] and colorectal adenocarcinoma [29].

Polymorphism in *EGF* gene from position – 1350 to 164 was characterized by Shahbazi et al. [30] where they identified a G to A substitution at position 61 in the 5′ untranslated region (UTR). The presence of the variant 61 A allele lead to a decreased *in vitro* EGF production in peripheral blood mononuclear cells. *EGF* G61G genotype was shown to be associated with the risk of developing malignant melanoma, gastric cancer [31, 32], glioma [33], hepatocellular carcinoma in cirrhotic patients [34], colorectal cancer [35] and recurrence of liver metastases from CRC [36].

In the present study we investigated the possible association of *p53* Arg72Pro and *EGF* A61G polymorphisms with CRC in the Slovak population. The analysis is supplemented by association of these single polymorphisms with clinicopathological features and tumour site. According to our knowledge this preliminary study is the first reporting possible association between the combined *p53* Arg72Pro and *EGF* A61G gene polymorphisms and colorectal cancer risk.

2. Material and Methods

2.1. Study population

Blood samples from 173 patients (mean age 66±12 years, range: 32-88 years) were histologically verified as having colorectal cancer. This group comprised patients who attended the Surgery Clinic and Oncology Centre of University Hospital in Martin in the period of November 2005 – December 2010. From the patients‘ medical records we obtained data on the age, date of diagnosis of CRC, clinical stage, tumour size, histological grade and type of tumour. The studied population is described in Table 1. Patients who had a hereditable gastrointestinal polyposis syndromes as well as CRC patients who presented in addition other cancers or other major pathologies were excluded. The control group comprised of 303 healthy volunteers who visited the general health check-up or medical and paramedical staff. The composition of the control group was comparable to the cases in terms of ethnicity (Caucasian only). Exclusion criteria for controls: blood relatives who had been diagnosed with CRC, their relationship to the respondent. Inclusion criteria was age >18 (mean age 52±13.8 years).

The present study was approved by the Ethical Board of Jessenius Faculty of Medicine, Comenius University and informed written consent was obtained from all individuals prior to the study.

2.2. Genotype analysis

Genomic DNA was isolated from peripheral leukocytes by proteinase K digestion (Applichem, Germany), phenol/ chloroform extraction and ethanol precipitation, dissolved in TE buffer (pH=7.5) and stored at -20°C until genotype analysis.

Genomic DNA (100 ng) was amplified in a total volume of 25 µl reaction mixture containing 25 pmol of the exon 4 *p53* gene sequence primers, (forward 5’-TTG CCG TCC CAA GCA ATG GAT GA-3’ and reverse 5’-TCT GGG AAG GGA CAG AAG ATG AC-3’ (synthesized by VBC-Biotech, Austria), and RedTaq ReadyMix PCR reaction mix (20 mM Tris-HCl, pH 8.3, with 100 mM KCl, 3 mM $MgCl_2$, 0.002% gelatin, 0.4 mM dNTP mix, stabilizers, and 0.06 unit/µl of Taq DNA Polymerase, Sigma-Aldrich, USA). After initial denaturation for 5 min at 94°C, 35 cycles were performed for 40 sec at 94°C

Table 1. Frequency distributions of selected variables in colorectal patients and cancer-free controls.

	Cases		Control		
Variable	No	%	No	%	p- value*
Age (years)					
<50	23	13.3	95	31.5	0.0001
51-60	43	24.9	113	37.4	ns
>61	107	61.8	94	31.1	ns
Sex					
Male	104	60.1	166	54.8	ns
Female	69	39.9	137	45.2	0.001
Site of tumour					0.005
Colon	82	47.4	n/a		
Rectosigmoideum	32	18.5	n/a		
Rectum	59	34.1	n/a		
Stage of tumours by TNM					ns
Stage 0-II	34	19.7	n/a		
Stage III-IV	90	52.0	n/a		
Incomplete clinical data	49	28.3	n/a		
Histological grade					ns
Grade I	3	5.0	n/a		
Grade II	38	63.3	n/a		
Grade III	19	31.7	n/a		
Lymph node metastasis					ns
Positive	21	18.6	n/a		
Negative	92	81.4	n/a		

TNM -the Tumour-Node-Metastasis classification, Grade I -well differentiated, grade II – moderately differentiated, grade III- poorly differentiated, anaplastic, or undifferentiated, n/a-not applicable, ns-not significant
**- Mann Whitney test or Kruskal-Wallis test*

(denaturation), for 30 sec at 68°C (annealing) and for 40 sec at 72°C (extension), followed by a final step of 10 min at 72°C. The PCR product was digested with 5 units of *BstUI* (New England, Biolabs) at 37°C for 16 hours. After digestion, the fragments were electrophoresed on 2% agarose gel and visualized by UV light after ethidium bromide staining. Thus, the proline (Pro72Pro) allele was identified by the presence of a single fragment of 199 bp and the arginine (Arg72Arg) allele by two fragments of 113 and 86 bp, respectively. Heterozygous (Arg72Pro) samples displayed all three fragments of 199 bp, 113 bp and 86 bp (Figure 1).

Genotyping of *EGF* was done by PCR-RFLP as described previously [30]. Briefly, the PCR primers used for amplifying *EGF* polymorphism were forward 5'-TGT CAC TAA AGG AAA GGA GGT-3' and reverse 5'-TTC ACA GAG TTT AAC AGC CC-3' (synthesized by VBC-Biotech, Austria). Reaction mixtures were preincubated at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 57°C for 1 min, 72°C for 1 min and a final extension step at 72°C for 10 min. The *EGF* amplification product of the size 242 bp was digested with 5 units of *Alu1* (New England, Biolabs) at 37°C for 16 hours. Restriction enzyme digestion products G61G (193 bp, 34 bp, and 15 bp), A61A (102 bp, 91 bp, 34 bp, and 15 bp), and A61G (193 bp, 102bp, 91 bp, 34 bp, and 15 bp) (Figure 1) were analyzed using the Shimadzu MCE-202 MultiNA microchip technology (Shimadzu Corporation, Kyoto, Japan) (Figure 1). The samples and reagents (separation buffer, DNA marker reagent, and 25 bp DNA ladder from DNA 500 kit on the Shimadzu MCE-202 MultiNA) were mixed automatically on-chip and ran using the MultiNA Control and MultiNA Viewer software.

2.3. Statistical analysis

Odds ratios (OR), 95% confidence intervals for OR and χ^2 test were used to test frequencies of genotypes/allele in CRC patients and controls. Hardy-Weinberg equilibrium was tested using a goodness-of-fit χ^2 test with one degree of freedom to compare observed genotype frequencies with expected genotype frequencies among

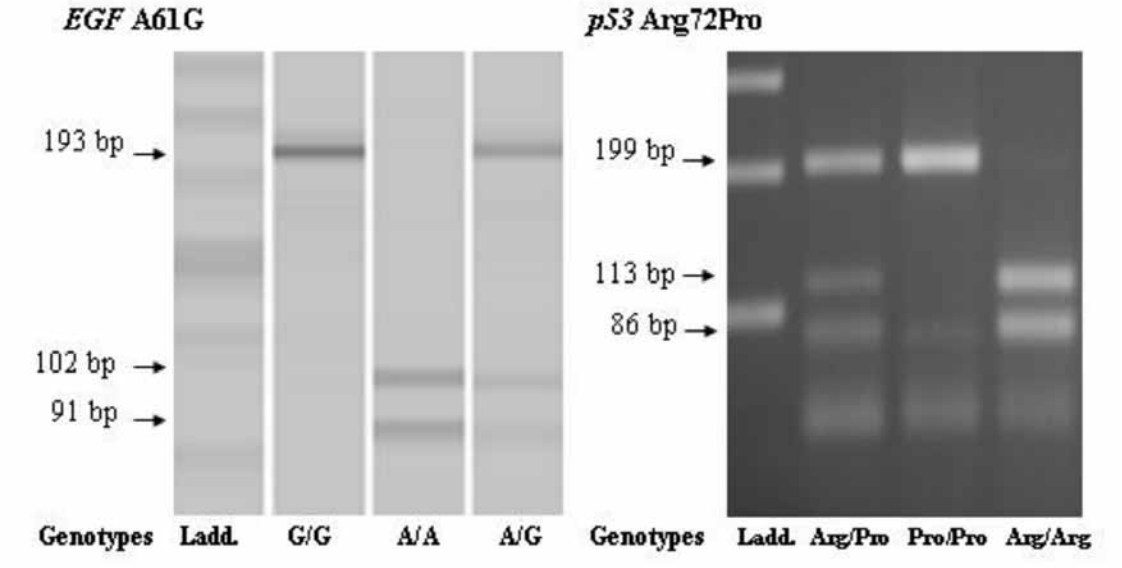

Figure 1. Product of PCR-RFLP analysis of EGF A61G (left) and p53 Arg72Pro (right) polymorphisms digested with AluI (MultiNa multichip electrophoresis) and Bst UI (agarose electrophoresis).

the subjects. We used unconditional logistic regression and simultaneous Entry method. Independent Variables: gender, *p53* and *EGF* genotypes, interaction between genotypes and age. Age was included in the models in three categories (<50, 51-60 and >61) when significant, to account for the matching. The statistical programs used were SPSS version 16.0 Software, Microsoft Excel and GraphPad Instat version 3.00 for Windows 95, GraphPad Software, San Diego California USA. All tests were two-sided and considered significant if $p<0.05$.

3. Results

3.1. Study population

The characteristics of the study population are presented in Table 1. In total, 173 cases and 303 controls were included in these analyses. Mann Whitney statistics tested no significant differences between age over 50 years and sex frequency distributions in men and control study groups. Statistically significant difference was found between groups less than age 50 years ($p<0.0001$) and female case and control study groups ($p=0.001$).

3.2. Genotypes of *p53* Arg72Pro and *EGF* A61G polymorphisms and CRC disease status

We examined the relationship between *EGF* and *p53* polymorphisms and histology, tumour grade, and metastasis at the time of diagnosis. Among 173 colorectal patients whose disease stages were determined, 34 (19.7%) had localized disease, defined as TNM stage 0, I or II, and 90 (52.0%) had advanced disease, defined as TNM stage III or IV. The remaining 49 (28.3%) patients had incomplete clinical data (Table 1). Surgically treated cancer cases involved the colon, rectum and rectosigmoid. Generally, colon and rectal cancer was more frequent in men in compariosn to women (48.4% vs 46.7% and 38.3% vs 28.1%, resp.) whereas rectosigmoid cancer was more frequent in women in comparison to men (23% vs 15%). Colon cancer was predominant to rectal cancer. There was significant association of age with a specific tumour site in total of cases and patients with rectal cancer tended to be younger than those with a colon cancer ($p=0.002$). The more advanced age of colon cancer patients (mean age 65±9.6 years) compared with rectal cancer (mean age 59±11.4 years) was primarily the result of age differences in male colon tumour patients ($p=0.007$) (data do not shown). Median age of male colon patients was 64 years whereas male rectum patients was age of 56 years. No such correlation between age and sex was found for any other tumour site.

No significant difference in genotype frequencies was found among tumour stages, grades or metastatic patients (Table 2). Assuming a dominant model, comparing early and late stage of disease, inverse association has been observed showing that *p53* Pro-72Pro genotype was more prevalent in CRC patients in early stages (I-II) (0.23, 95% CI 0.06-0.87, $p=0.03$). In the dominant model that compares early stage CRC patients to healthy subjects has shown a positive

association between early onset of the disease and *p53* Pro72Pro genotype (3.8, 95% CI 1.39-10.42, p=0.009) (data not shown).

Stratification analysis using χ2 and Fisher´s exact tests (if needed) revealed a strong effect of gender on the association of *p53* Arg72Pro and *EGF* A61G polymorphisms with different CRC site in only female patients given in Tables 4 and 5. Significant difference between female colon cancer cases and controls was found for *p53* Pro72Pro genotype compared with Arg72Arg (4.0; 95% CI 1.27-12.7; p=0.04). In contrast, non significant association was observed for Pro72Pro and reduced risk of female rectal cancer (0.67; 95% CI 0.08-5.89) in comparison to non significant association with increased risk of rectal cancer in men (2.63; 95% CI 0.6-11.47). However, the *p53* homozygous Pro allele (Pro72Pro genotype) showed to be non significantly protective against colon and rectosigmoid junction cancer in men patients (0.53; 95% CI 0.06-5.04 and 0.61; 95% CI 0.03-12.4, respectively). Similarly, the genotype EGF G61G showed non significant protective effect in respect to tumour site in men (Table 4) but poses a potential risk factor for CRC in women, mainly in rectosigmoid junction cancer (4.5; 95% CI 1.2-16.9; p=0.02).

3.3. Prevalence of *p53* Arg72Pro genotypes in CRC patients

No significant deviation from expected genotype frequencies under Hardy-Weinberg equilibrium were observed in the total control group. However, *p53* genotype distribution of the men control group was not in

Table 2. Associations between the p53 Arg72Pro or EGF A61G polymorphism and clinical characteristics.

Variable	p53 Arg72Pro				EGF A61G			
	Arg/Arg	Arg/Pro	Pro/Pro		A/A	A/G	G/G	
	N (%)	N (%)	N (%)	p-value	N (%)	N (%)	N (%)	p-value
Site of tumour								
Colon	39 (48.2)	35 (42.2)	8 (9.6)	ns	31 (37.3)	39 (48.2)	12 (14.5)	ns
Rectosigmoideum	16 (50)	15 (46.9)	1 (3.1)		9 (27.3)	20 (63.6)	3 (9)	
Rectum	33 (55.9)	21 (35.6)	5 (8.5)		18 (31.6)	30 (50.9)	10 (17.5)	
Stage of tumours by TNM								
Stage 0-II	16 (47.1)	12 (35.3)	6 (17.6)	ns	15 (44.1)	13 (38.2)	6 (17.7)	ns
Stage III-IV	46 (51.1)	39 (43.3)	5 (5.6)		29 (32.2)	48 (53.3)	13 (14.5)	
Histological grade								
Grade I	1 (33.3)	1 (33.3)	1 (33.3)	ns	3 (100.0)	0 (0.0)	0 (0.0)	ns
Grade II	21 (55.3)	14 (36.8)	3 (7.9)		15 (39.5)	19 (50.0)	4 (10.5)	
Grade III	10 (52.6)	8 (42.1)	1 (5.3)		9 (47.4)	8 (42.1)	2 (10.5)	
Lymph node metastasis								
Positive	7 (33.3)	10 (47.6)	4 (19.1)	ns	6 (28.6)	10 (47.6)	5 (23.8)	ns
Negative	46 (50.0)	38 (41.3)	8 (8.7)		35 (38.0)	44 (47.8)	13 (14.2)	

Table 3. Frequency distribution of *EGF* A61G or *p53* Arg72Pro polymorphism between cases and controls and its association with risk of colorectal cancer

Genotypes	Cases (n=177)		Controls (n=313)		OR (95% CI)*	p- value
	No (%)		No (%)			
p53 Arg72Pro						
Arg/Arg	90	51.1	159	50.5	0.25 (0.04-1.6)	ns
Arg/Pro	72	40.9	138	43.8	0.14 (0.02-0.99)	0.05
Pro/Pro	14	8	18	5.8	1.00 (Reference)	
EGF A61G						
A/A	59	33.9	115	36.7	0.21 (0.02-1.9)	ns
A/G	90	51.4	136	43.5	0.47 (0.05-4.24)	ns
G/G	26	14.9	62	19.8	1.00 (Reference)	

** OR adjusted for age and gender*

Table 4. Frequency distribution of p53 codon 72 polymorphism between cases and controls and its association with risk of colorectal cancer or selected diagnosis.

p53 Arg72Pro	Arg/Arg	Arg/Pro	Pro/Pro	OR (95% CI)	p- value
Men					
Controls	79 (46.7%)	84 (49.7%)	6 (3.6%)	1.00 (Reference)	
All cases	58 (56.2%)	41 (39%)	5 (4.8%)	1.12 (0.32–3.8)	ns
Colon	26 (54%)	22 (44%)	1 (2%)	0.53 (0.06–5.04)	ns
Rectosigmoideum	10 (66.7%)	5 (33.3%)	0 (0%)	0.61 (0.03–12.4)	ns
Rectum	22 (55%)	14 (35%)	4 (10%)	2.63 (0.60–11.47)	ns
Women					
Controls	80 (54.8%)	54 (37%)	12 (11.6%)	1.00 (Reference)	
All cases	30 (43.7%)	30 (43.7%)	9 (12.7%)	1.94 (0.7–5.1)	ns
Colon	13 (39.4%)	13 (39.4%)	7 (21.2%)	4.0 (1.27–12.7)	0.04
Rectosigmoideum	6 (35.3%)	10 (58.8%)	1 (5.9%)	1.24 (0.13–11.5)	ns
Rectum	11 (57.9%)	7 (36.8%)	1 (5.3%)	0.67 (0.08 -5.89)	ns

Table 5. Frequency distribution of EGF A61G polymorphism between cases and controls and its association with risk of colorectal cancer or selected diagnosis.

EGF A61G	A/A	A/G	G/G	OR (95% CI)	p- value
Men					
Controls	52 (30.6%)	76 (44.7%)	42 (24.7%)	1.00 (Reference)	
All cases	35 (33.7%)	55 (52.8%)	14 (13.5%)	0.48 (0.2–0.9)	0.04
Colon	18 (36%)	25 (50%)	7 (14%)	0.59 (0.22–1.58)	ns
Rectosigmoideum	6 (37.5%)	9 (56.3%)	1 (6.3%)	0.25 (0.03–2.2)	ns
Rectum	10 (26.3%)	22 (57.9%)	6 (15.8%)	0.9 (0.30–2.7)	ns
Women					
Controls	63 (44%)	60 (42%)	20 (14%)	1.00 (Reference)	
All cases	22 (33.3%)	31 (47%)	13 (19.7%)	1.86 (0.80–4.36)	ns
Colon	13 (39.4%)	15 (45.5%)	5 (15.2%)	1.2 (0.37–3.8)	ns
Rectosigmoideum	3 (17.6%)	12 (70.6%)	2 (11.8%)	4.5 (1.2 -16.97)	0.02
Rectum	8 (42.1%)	7 (36.8%)	4 (21%)	1.56 (0.42–5.83)	ns

agreement with Hardy-Weinberg equilibrium (χ^2=8.34, p=0.005) due to possibility of selection bias from control group that were not random samples from the general population. Table 3 shows the frequencies of *p53* codon 72 genotypes in colorectal case and control group. The analysis of the DNA from the CRC patients revealed 14 (8%) proline homozygotes (Pro72Pro), 72 (40.9%) arginine72proline heterozygotes (Arg72Pro), and 90 (51.1%) arginine homozygotes (Arg72Arg). The proportions found in control populations were *p53* 50.5% arginine homozygous, 43.8% heterozygous and 5.8% proline homozygous.

Logistic analysis showed significant association between cases and controls for *p53* Arg72Pro genotype compared with Pro72Pro (0.14; 95% CI 0.02-0.99, p=0.05) (Table 3).

We used unconditional logistic regression analysis to find the relationship between several risk factors and probability of disease development. Five independent variables were used: age, gender, *EGF* A61G and *p53* Arg72Pro genotypes and interaction between genotypes. The analysis showed significant association between age, *p53* Agr72Pro genotype and risk of CRC (0.17; 95% CI 0.11-0.26, p=0.001) (data do not shown). These results suggest that genotype Arg72Pro as well as younger patients have decreased CRC risk in comparison to Pro72Pro and older age group of patients.

3.4. Prevalence of *EGF* A61G genotypes in CRC patients

Genotype frequencies of A61G polymorphism in *EGF* gene are listed in Table 3. Men have been found to have greater chance of the disease (p=0.018) in comparison to women by using logistic regression model (data not shown). Table 5 shows subgroup analysis by gender and

tumour site indicating that the genotype *EGF* G61G was inversely associated with significantly increased risk of colorectal cancer in men patients by χ^2 and Fisher´s exact tests (0.44; 95% CI 0.2-0.9; p=0.04).

Although larger number of patients are clearly needed to verify this we suggest that genotype *EGF* G61G may be a potential protective factor in men with diagnosted colorectal cancer.

3.5. Association between both genetic variants and colorectal cancer risk

We evaluated further whether susceptible genotypes for *p53* Arg72Pro and *EGF* A61G are together associated with an increased risk of CRC. As shown in Table 6, the variant genotypes of *p53* Arg72Pro and *EGF* A61G were associated with a significantly increased risk of CRC among carriers with *EGF* G61G (5.57; 95% CI 1.11-27.9; p=0.05 for *p53* Pro72Pro) by using chi-squared or Fisher`s exact tests. Interestingly, we have observed that risk of CRC is the 2.94 less likely in patients carrying G61G/Arg72Pro genotype (0.34 95% CI 0.14-0.83, p=0.02). In subgroup analysis by gender, the combination of genotypes *EGF* G61G and *p53* Pro72Pro were associated with an increased CRC risk in men (6.3; 95% CI 0.69-57.52, p=0.08) but not in women and seems to be important in the development of the disease. The combined genotype G61G/Arg72Pro has been shown to be beneficial to men with CRC (0.22; 95% CI 0.06-0.77, p=0.02). No significant association was shown between combined genotypes of both polymorphisms and TNM staging, lymph node metastasis or histopatological grading and tumour site (data not shown).

4. Discussion

In our study we have investigated the contribution of independent and the combined effects of the *EGF* A61G and *p53* Arg72Pro polymorphisms to CRC risk in Slovak population. According to our knowledge this is the first study showing a possible combined association of genetic polymorphic variants of the *p53* and *EGF* genes with CRC risk.

Our study has shown several interesting findings. We report the association between *p53* Pro72Pro genotype and susceptibility to CRC risk in Slovak population. Previous studies have implicated that both Arg and Pro alleles may be associated with the higher risk of malignancy. The risk of tumour progression in CRC patients considerably vary between racial and ethnic groups [6,13,17,18]. We found that frequency of genotype

Table 6. Number of all cases and controls, ORs, 95% CIs, $\chi 2$ and P by TP53 Arg72Pro and EGF A61G

p53 Arg72Pro	EGF A61G	Cases (n=177) No (%)		Controls (n=303) No (%)		OR (95% CI)	p- value
Arg/Arg	A/A	35	(20.2%)	68	(21.8%)	0.9(0.58-1.44)	ns
Arg/Arg	A/G	39	(22.5%)	59	(18.9%)	1.2(0.79-1.97)	ns
Arg/Arg	G/G	14	(8.1%)	29	(9.3%)	0.85(0.44-1.67)	ns
Arg/Pro	A/A	21	(12.1%)	37	(11.9%)	1.03(0.58-1.8)	ns
Arg/Pro	A/G	44	(25.4%)	71	(22.8%)	1.16(0.75-1.79)	n
Arg/Pro	G/G	6	(3.5%)	30	(9.6%)	0.34(0.14-0.83)	0.02
Pro/Pro	A/A	3	(1.7%)	10	(3.2%)	0.53(0.14-1.96)	ns
Pro/Pro	A/G	5	(2.9%)	6	(1.9%)	1.52(0.46-5.05)	ns
Pro/Pro	G/G	6	(3.5%)	2	(0.6%)	5.57(1.11-27.9)	0.05
Men							ns
Arg/Arg	A/A	22	(20%)	27	(15.98%)	1.3(0.71-2.45)	ns
Arg/Arg	A/G	25	(22.7%)	31	(18.3%)	1.3(0.72-2.37)	ns
Arg/Arg	G/G	9	(8.2%)	21	(12.4%)	0.63(0.28-1.43)	ns
Arg/Pro	A/A	14	(12.7%)	22	(13%)	0.97(0.48-1.99)	ns
Arg/Pro	A/G	28	(25.5%)	43	(25.4%)	1.0(0.58-1.74)	ns
Arg/Pro	G/G	3	(2.7%)	19	(11.2%)	0.22(0.06-0.77)	0.02
Pro/Pro	A/A	1	(0.9%)	3	(1.8%)	0.51(0.05-4.95)	ns
Pro/Pro	A/G	4	(3.6%)	2	(1.2%)	3.1(0.56- 17.51)	ns
Pro/Pro	G/G	4	(3.6%)	1	(0.6%)	6.3(0.69–57.52)	ns

Product of PCR-RFLP analysis of EGF A61G (left) and p53 Arg72Pro (right) polymorphisms digested with AluI (MultiNa multichip electrophoresis) and Bst UI (agarose electrophoresis).

p53 Arg72Arg in healthy population was 51.1%, and in cases 50.5% and mutant Pro72Pro frequency was 5.8% in controls and 8% in CRC patients. Distributions of *p53* Arg72Pro genotypes in our healthy control group are almost identical to those reported for the Croatia, Germany, Italy, and other nearby countries, comparable in ethnicity and latitude [37-41]. Chi-square or Fischer exact tests did not reveal association between the CRC risk and *p53* Arg72Pro polymorphism. However, unconditional logistic regression with independent variables like age, gender and both of genotypes built a model by removing non-significant factors and keeping only age and *p53* Arg72Pro genotypes as the significant interacting factors (p=0.001) relate to CRC risk. Out of 173 CRC patients, 85% were over 60-years old and only 15% were less than 60 years of age. Genotype *p53* Arg72Pro as well as younger patients have decreased CRC risk in comparison to Pro72Pro and older age group of patients (0.14; 95% CI 0.02-0.99, p=0.049). Our results are consistent with the recent published studies [12,13, 42-44]. Few other studies have reported contradictory findings, e.g. Tang et al. [45] conducted a meta-analysis involving 17 case control studies with a total of 3537 CRC cases and 5168 controls as study subjects. They did not find any significant association of *p53* Pro72Pro genotype with CRC risk. Similarly, Economopoulos et al. [19] did not find any significant risk association when they provided a meta-analysis study involving 19 Caucasian, 6 Chinese and 2 mixed populations. The difference in results on risk association between the present study and the above mentioned might be due to difference in groups studied or populations, and also differences in environmental exposure, lifestyle factors, tissue and age specificity. The complex of lifestyle endo- and exogenous factors of each ethnic group, the proportion of which increases with age, may dramatically modulate the contribution of *p53* polymorphisms to cancer risk through, for example, genotoxic effects and epigenetic modifications of the *p53* gene structure. Exogenous modifiable factors, such as alcohol, smoking and betel or areca quid chewing, and radiation and chemical poisoning, together with endogenous estrogen metabolites and other secreted chemicals, have been found to be involved in DNA damage and epigenetic alterations [46 and citations there].

The current study provides additional knowledge regarding the association of the investigated polymorphisms and CRC in relation to the colorectal tumour site. The basic data on overall incidence of CRC in relation to age, gender and anatomical location were similar to those reported from other studies [47-49]. Our results showed predominance of colon cancer over rectal cancer site (p=0.002) in patients. Different molecular alterations have been implicated in carcinogenesis of sporadic cancers of colon, rectosigmoid junction and rectum. Existing data suggest different prognosis and outcome leading to a need for individualized therapy [50]. Two distinct genetic pathways have been proposed in the development of CRC. Chromosomal instability that leads to a progression from normal mucosa to adenoma and carcinoma, is more frequent in distal CRC including rectal cancer. Mutations accumulate in the Kras oncogene and tumour suppressor genes, including APC on 5q, *p53* on 17p, and *SMAD4* on 18q [51]. The second pathway is characterized by mutations in mismatch-repair genes and the following microsatellite instability is responsible for proximal cancer [52]. The present study showed increased risk of Pro carrier genotypes with colon cancer in women (4.0; 95% CI 1.27-12.7, p=0.04) but not in men. Similar results were observed in case control study of Koushik et al. [53]. They showed a moderately increased risk of Pro carrier genotypes with proximal colon cancer in women and distal colon cancer in men. On the contrary the Greece-Caucasian study suggested a positive association between Arg homozygous carriers and left colon cancer including descending, sigmoid and rectum (2.98; 95 CI 1.15-7.7, p=0.026) in both sexes [54]. The other case control study did not show any association to tumour site [55]. More studies with larger population groups and more evidence are required to understand differences between the molecular biology of colon, rectosigmoid and rectal cancers.

The EGFR system is an important mediator within the tumour microenvironment of autocrine and paracrine circuits leading to dysregulated EGFR activation and uncontrolled tumour growth. In this regard, many methods were developed to detect commonly known mutations and to screen new mutations of the EGFR in CRC but mainly in non-small cell lung cancer [28]. Epidermal growth factor EGF is frequently found co-expressed with EGFR in various types of cancer including colorectal adenocarcinoma [29]. In addition, significant regional differences in EGFR expression in the normal human colon mucosa was previously found. The EGFR level was significantly higher in samples from the proximal part of the normal colon than they was in samples from the distal part (p<0.05). The EGFR levels of the colorectal carcinoma samples did not show any regional variation [56]. In another report, the significant increase in EGFR level as well as growth factors including EGF was observed in the left-sided colon [29] of patients with colorectal adenocarcinoma. These experiments suggest different growth properties in the proximal and distal colon of normal and neoplastic colon tissue. EGF promoter polymorphisms were observed to modulate

EGF levels and thought to have effect on susceptibility to various carcinomas but the results are contradictory [30,57-58]. In the present study we observed a significant association of *EGF* G61G genotype with rectosigmoid junction and rectal cancers (1.74, 95% CI 1.018-3.0, p=0.04) in total cases and especially in women (4.5, 95% CI 1.2-16.9, p=0.02). There was not significant association between *EGF* A61G genotype and tumour site in CRC male patients. Although EGF level in normal or neoplastic colon was not assessed in the present study we suppose on the basis of previously published studies that there will be differences between three different *EGF* A61G genotypes in CRC patients with different tumour site and gender. Our analysis of *EGF* A61G genetic polymorphism showed the unexpected significant inverse association of variant genotype *EGF* G61G with risk of CRC (0.44, 95% CI 0.2-0.9, p=0.04) in men and may serve as a potential protective factor. However, unconditional logistic regression, which adjusted for age and gender, did not confirm this correlation; this may be due to insufficient sample size and missing data in logistic analysis. Recently, it has been shown that the *EGF* G61G genotype is associated with improved overall survival and progression-free survival or less tumour recurrence in patients with metastatic CRC treated with cetuximab-irinotecan salvage therapy [59,60] or patients with esophageal cancers [58,61]. The possible explanation of these results is the different effect of increased EGF activity in different stages of cancer development and possibility of EGF-inducing apoptosis or increase of cancer susceptibility to treatment. In this respect, recent studies showing the complete pathological response of advanced rectal cancer patients treated with cetuximab-based neoadjuvant chemoradiation were strongly associated with *EGF* G allele suggesting possible enhanced inhibition of EGFR pathway or increased sensitivity to radiation therapy [62]. The protective role of *EGF* 61G allele may suggest also possible influence of the 61A allele that might be masked by the presence of other as-yet unidentified causal genes involved in CRC development. In present study, combined analysis showed that the variant genotypes of *p53* Arg72Pro and EGF A61G were associated with a significantly increased risk of CRC among carriers with *EGF* G61G (5.57; 95 % CI 1.11-27.9, p=0.05 for *p53* Pro72Pro) without adjustment for age and gender. However, the association signal was weak and based on a limited number of individuals. It seems that only increased degradation of variant *p53* Pro72 homozygous individuals may result in the EGF G61G variant becoming a risk allele, preferentially in men. We observed that the CRC risk is 2.94 times less likely in patients carrying genotype G61G/Arg72Pro (0.34, 95% CI 0.14-0.83, p=0.02). Since the combined genotype G61G/Arg72Pro has been shown to be beneficial mainly to men with CRC (0.22; 95 % CI 0.06-0.77, p=0.02) we suppose that low apoptotic capacity of p53 Pro72Pro and stronger ability of induction the transcription and cell cycle arrest make for potential pro-oncogenic effects mediated by the *EGF* G61G. Unfortunately, unconditional logistic regression did not show association of both the genotypes with CRC after adjustment for age, genotypes and gender but this may be due to the small sample size and the possible selection bias because all of the patients were treated at a single hospital. Thus, further confirmatory studies in larger cohorts are necessary to ascertain such epistatic polymorphic effects. Influence of confounding factors such as smoking and alcohol consumption, dietary habits, could not be determined in this study, as detailed information could not be obtained from clinical charts. In conclusion, our study provides evidence that *p53* Arg72Pro polymorphism may contribute to the etiology of CRC in the Slovak population and individuals who are above 60 years old.

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Conflict of interest statement

Authors state no conflict of interest.

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