Full Length Research Paper

Association of the *p53* codon 72 polymorphism with colorectal cancer in South West of Iran

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The *p53* tumor suppressor gene plays two important roles in genomic stability: blocking cell proliferation after DNA damage until it has been repaired, and starting apoptosis if the damage is too critical. Codon 72 exon4 polymorphism (*Arg72Pro*) of the *P53* gene has been implicated in cancer risk. Various studies have been done to investigate the status of *p53* at codon 72 for arginine (*Arg*) and proline (*Pro*) alleles in different populations and also the association of this codon 72 polymorphism with various tumors. Our objective was to investigate the possible association between *P53 Arg72Pro* polymorphism and susceptibility to colorectal cancer among Isfahan and Chaharmahal Va Bakhtiari (a part of south west of Iran) population. We investigated the status of *p53* at codon 72 for *Arg/Arg, Arg/Pro* and *Pro/Pro* allele polymorphisms in blood samples from 145 colorectal cancer patients and 140 controls by Nested-PCR of *p53* exon 4 and digestion with *BstU*I restriction enzyme and the DNA fragments were then resolved by electrophoresis in 2% agarose gel. The *Pro* allele was 279 bp, while the *Arg* allele was restricted into two fragments of 160 and 119 bp. Among the 145 colorectal cancer cases 49 cases (33.79%) were homozygous for the *Arg72* allele (*Arg/Arg*), 18 cases (12.41%) were homozygous for the *Pro72* allele (*Pro/Pro*) and 78 cases (53.8%) found in heterozygous (*Arg/Pro*). In conclusion, it can be said that *p53Arg/Arg* genotype may be correlated with possible increased risk of this kind of cancers in south west of Iran.

Key words: Colorectal cancer, nested-PCR, *p53 codon 72*, polymorphism, Iran.

INTRODUCTION

Colorectal cancer (CRC) also called colon cancer or large bowel cancer, includes cancerous growths in the colon, rectum and appendix (Zhu et al., 2007). It is the third most common cancer worldwide, the second leading cause of cancer death in the United States after lung cancer and the significant cause of morbidity and mortality in western populations (Steele, 2006).

Colorectal cancer is a complex, multistep and multifactor process, and is thought to result from an interaction between environmental and genetic factors (Gryfe et al., 1997). The risk of developing colorectal cancer is influenced by a number of factors, including sex, age, alcohol consumption, low-fiber or high-fat diet intake hereditary conditions, familial history of colorectal cancer, personal history of colonic polyps, bowel inflammatory diseases and a variety of genetic factors (Gryfe et al., 1997). Among the genetic factors, the *TP53* tumor suppressor gene is a suitable candidate for modulating colorectal cancer risk and is one of the most commonly mutated genes in all types of cancer (found in 50% of human cancers) (Schneider et al., 2004; Zhu et al., 2007). The *p53* gene located on chromosome 17p13 (Fan et al., 2000). It contains 11 exons and encodes for a

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Abbreviations: RFLP, Restriction fragment length polymorphism; *Tp53*, tumor suppressor gene p53; **CRC,** colorectal cancer.

Table 1. Prevalence of colorectal cancer cases according to racial groups.

Age group	Male		Female		Total	
	N	%	N	%	N	%
30>	4	2.77	5	3.44	9	6.2
30-40	9	6.21	7	4.82	16	11.03
40-50	16	11.05	13	8.95	29	20
50-60	13	8.98	17	11.7	30	20.7
60<	35	24.19	26	17.89	61	42.07
Total	77	53.2	68	46.8	145	100

53 kDa multifunctional DNA-binding protein (Oren, 1999). The *p53* plays a critical role in the prevention of tumor formation; however, it is not required for normal cell growth. *p53* can induce cell cycle arrest, DNA repair, differentiation, or apoptosis in response to oncogenic cellular stress including carcinogen induced DNA damage, abnormal proliferative stimulation (Lung et al., 2004). In the cell, *p53* protein binds DNA, which in turn stimulates another gene to produce a protein called p21 that interacts with a cell division-stimulating protein (cdk2). When p21 is complexed with cdk2 the cell cannot pass through to the next stage of cell division. Mutant *p53* can no longer bind DNA in an effective way, and as a consequence the p21 protein is not made available to act as the 'stop signal' for cell division. Thus cells divide uncontrollably, and form tumors (Steele, 2006). *p53* gene is the target of point mutations and of small deletions and insertions (Soong et al., 2000). These mutations (mostly missense mutations) may damage the normal function of *TP53* as a transcription factor, and the induction of repair or apoptosis may be abolished. Consequently, other genetic alterations may accumulate in the cell (Langerod et al., 2002).

To date, several polymorphisms in the wild-type *p53* gene locus have been described (Ara et al., 1990). At least three of them have been reported to be involved in cancer: the 16-bp duplication in Intron 3, MspI restriction fragment length polymorphism (MspI-RFLP) in Intron 6 (cc) and an arginine/praline variant at Codon 72 in Exon 4 (Khrunin et al., 2005; Mammano et al., 2009). The most important *p53* polymorphism is the *BstU*I-RFLP in Codon 72 of Exon 4 results in two alleles, the arginine (72Arg: CGC) and proline (72Pro: CCC) isoforms (Gryfe et al., 1997; Onrat et al., 2009). This polymorphism has been reported to be associated with some tumor types [Pro: bladder cancer, lung cancer; Arg: hepatocellular carcinoma, cervical cancer, human papilloma virus (*HPV*)-associated cervical cancer]. An alteration of the *p53* tumor suppressor gene is also a mutational event involved in colorectal mucosa carcinogenesis (Onrat et al., 2009; Schneider et al., 2004).

The *Arg72Pro* polymorphism is located in a proline rich region (residues 64-92) of the *p53* protein, where the 72Pro amino acid constitutes one of five PXXP motifs resembling a SH3 binding domain. The region is required for the growth suppression and apoptosis mediated by *p53* but not for cell cycle arrest (Onrat et al., 2009; Langerod et al., 2002). The p53Arg72 and p53Pro72 proteins do not differ in their ability to bind to DNA but they have some different biochemical and biological properties such as different binding to components of the transcriptional machinery and different activation of transcription (Langerod et al., 2002; Perez et al., 2006). In this sense, it appears that this polymorphism may be associated with differential susceptibility to cancer (Perez et al., 2006). The p53Arg72 protein induces apoptosis faster and suppresses transformation more efficiently than the *p53Pro72* protein (Lung et al., 2004). Conversely, the *p53Arg72* protein is more susceptible to proteolysis mediated by the E6 protein of the human papillomavirus and degradation of *p53* protein by HPV E6 is correlated with increased risk for HPV-associated cancers (Lung et al., 2004; Schneider et al., 2004). Although the presence of HPV-DNA in colorectal tissues and adenocarcinomas was reported in populations from different regions, the association of *p53* codon 72 polymorphism with colorectal cancer taking into account HPV infection was investigated only once (Sayhan et al., 2001). Therefore, we investigated the association of *p53* codon 72 polymorphism with colon cancer cases in south west of Iran by Nested-PCR and RFLP analysis with *BstU*I restriction enzyme.

MATERIALS AND METHODS

Subjects

A total of 145 patients (77 patients (53%) were male and 68 (47%) were female) with colorectal cancer, who attended the clinical hospitals and clinical laboratories from south west of Iran were enrolled into this study. Their age ranged from 18 to 85 years with a mean of 48.56 years (Table 1). Informed consent was obtained from the patients and their blood samples were collected. Blood from 140 people who attended clinical laboratories and were negative for colorectal cancer served as normal control.

DNA extraction from blood samples

Genomic DNA was extracted from the blood samples by using a DNA extraction kit (QIAGEN Ltd., Crawley, UK) in accordance with
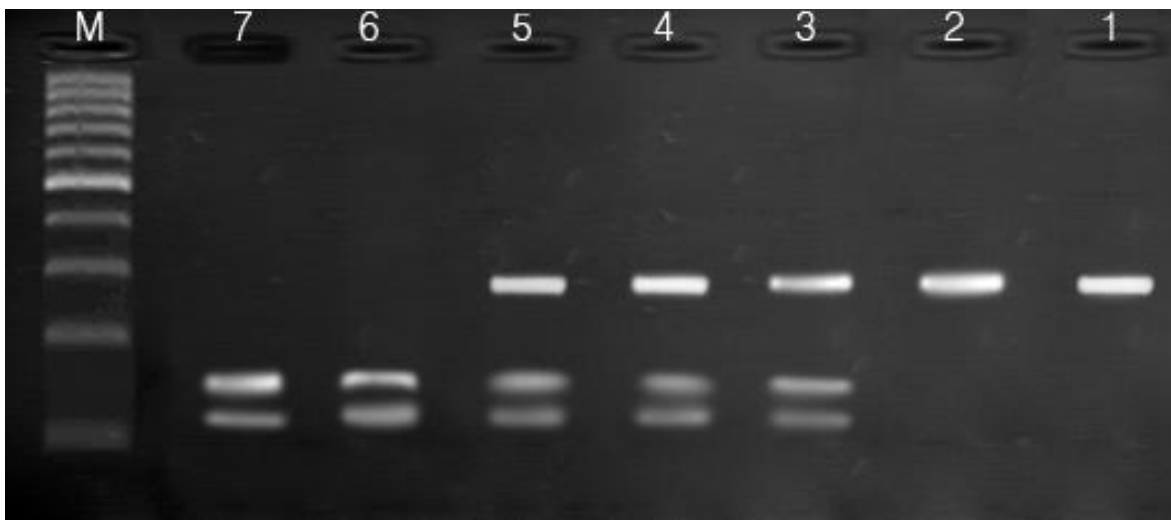
Figure 1. Gel electrophoresis of nested-PCR product after digestion with *BstU*I enzyme. Line M is 100 bp DNA ladder (Fermentas, Germany); Lines 1 and 2 are homozygote samples for *Arg* allele, Lines 3 to 5 are *Arg/Pro* heterozygote, and Lines 6 and 7 are *Pro* homozygote samples.

the manufacturer's protocol. The extracted DNA was stored at -20°C until further use.

p53 codon 72 polymorphism analysis

Nested-PCR and RFLP analyses were used to genotype the G-to-C *p53* polymorphism in Codon 72 as described previously.

Gene amplification with Nested-PCR method

The first PCR primers used were D1-F: 5′-GCT CTT TTC ACC CAT CTA CAG- 3′ and D2-R: 5′-TGA AGT CTC ATG GAA GCC AGC- 3′. PCR was performed in a final volume of 25 µl reaction buffer containing 20 ng genomic DNA, 2 mM MgCl$_2$, 25 pmole of each primer (D1-F and D2-R), 1 unit of Taq polymerase, 200 mM dNTP mix. Reaction mixtures were preincubated at 95°C for 5 min, followed by 30 cycles at 94°C for 1 min, 58°C for 1 min, 72°C for 1 min and a final extension step at 72°C for 5 min. Reaction mixture without DNA template was used as a negative control and that with known DNA template was used as a positive control which yielded PCR products of expected results.

The second PCR primers used were F2-F: 5′-TCC CCC TTG CCG TCC CAA- 3′ and R2-R: 5′-CGT GCA AGT CAC AGA CTT- 3′. Reaction mixtures were as above, except that 2.5 µl of product from the first reaction as template DNA and the second primers set were used.

RFLP analysis

The amplified fragments from second PCR were purified and digested with *BstU*I restriction enzyme (Fermentas, Germany) according to the instructions of the manufacturer. Each digestion reaction mixture (30 µl) contained 10 units of *BstU*I endonucleas and 25 µl of second PCR products and was incubated at 37°C for 2 h. After digestion, the fragments were electrophoresed on 2% agarose gel and visualized by UV light after ethidium bromide staining.

Statistical analysis

Analysis of data was performed using the SPSS version 17.0 computer software (SPSS, Chicago, IL). Chi-square analysis was used to compare categorical variables. A 5% level of significance was used in the analysis and its 95% confidence interval (CI) was calculated to measure the association between *P53 Arg72Pro* polymorphism in case and control population. Whenever appropriate, the observed number of each genotype was compared with that expected for a population in the Hardy-Weinberg equilibrium by using a goodness of fit χ^2 tests.

RESULTS

Amplified DNA fragments with *Arg* at Codon 72 would contain the polymorphic *BstU*I site thus the *Pro* allele was 279 bp, while the *Arg* allele was restricted into two fragments of 160 and 119 bp. Among the 145 colorectal cancer cases 49 cases (33.79%) were homozygous for the *Arg 72* allele (*Arg/Arg*), 18 cases (12.41%) were homozygous for the *Pro72* allele (*Pro/Pro*) and 78 cases (53.8%) found in heterozygous (*Arg/Pro*). The frequencies of the three *P53* genotypes; *Arg/Pro*, *Arg/Arge* and *Pro/Pro* in controls were 60.7, 20 and 19.3%, respectively.

A representative nested-PCR and RFLP pattern is depicted in Figure 1. The results on genotype distribution are presented in Table 2.

In this regard, an increase in the *Arg/Arg* genotype and

Table 2. Distribution of *p53 codon 72* polymorphism genotypes among colorectal cancer cases and controls in south west of Iran.

Genotype	Cases (N=145)		Controls (N=140)		P-value
	N	**%**	**N**	**%**	
Arg/Arg	49	33.79	28	20	<0.01
Arg/Pro	78	53.8	85	60.7	<0.02
Pro/Pro	18	12.41	27	19.3	>0.005

Table 3. Allelic frequencies of *TP53 codon 72* among colorectal cancer cases and controls in south west of Iran.

Alleles	Cases frequency		Controls frequency		P-Value
	N	**%**	**N**	**%**	
Arg	88	60.69	71	50.35	>0.10
Pro	57	39.31	69	49.65	<0.05

a decrease in *Pro/Pro* genotype among patients was observed, and have a statistical significance (P<0.01). Comparison of the allele frequencies revealed an increase in the *Arg* allele among colorectal cancer patients compared to healthy individuals (Table 3). However, the difference was not statistically significant (P = 0.10). Comparison of the genotype frequencies between colorectal cancer patients and normal controls confirmed the accumulation of *Arg/Arg* genotype in these patients (33.79 vs. 20%, P<0.01).

DISCUSSION

The *p53* gene is one of the most extensively studied human genes because of its role as a tumor suppressor gene. The overall function of *p53* is to maintain genomic integrity as a whole, providing a protective effect against tumorigenesis (Onrat et al., 2009). Recently, several studies have provided evidence that alterations of *p53*, either through genetic mutation or allelic polymorphisms, have been described as the most common genetic changes in human cancers, such as cervical cancer, breast cancer, lung and colorectal cancer (Lung et al., 2004; Onrat et al., 2009). In Iran colorectal cancer accounts are approximately 8% of all malignancies (Mojtahedi et al., 2010). The *p53* gene is known to exhibit distinct mutational patterns in various cancer types. The proline/arginine substitution at Codon 72 represents a common amino acid polymorphism in the *p53* gene, the functional significance of which is unknown (Onrat et al., 2009). This may be due to the production of a mutant *p53* protein that is not capable of inducing apoptosis in response to toxic environmental stimuli (Lung et al., 2004). The role of the *Arg/Pro* polymorphism in colorectal cancer susceptibility has been examined in several studies, which have reported controversial results (Nikbahkt et al., 2008). This controversy might be due to

the fact that the genotype distribution of the *p53 codon 72 Arg/Pro* polymorphism varies in different geographic regions and ethnicity (Mojtahedi et al., 2010). According to the literature, general populations from Latin America, United States and Europe exhibit high frequencies of the *Arg* allele compared to the *Pro* one, while lower prevalence's of *Arg* are found in African and Asian populations (Pignatelli et al., 1992).

Relevant to this, by nested-PCR, amplification of 4th exon were performed from isolated DNA samples of blood of colon cancer diagnosed in 145 cases and followed by enzyme restriction. As a result, the analysis of the *p53* codon 72 genotypes revealed 49 cases (33.79%) arginine homozygotes (*Arg/Arg*), 18 cases (12.41%) proline homozygotes (*Pro/Pro*) and 78 cases (53.8%) arginine/proline heterozygotes (*Arg/Pro*). The frequencies of the three *P53* genotypes; *Arg/Pro*, *Arg/Arge* and *Pro/Pro* in controls were 60.7, 20 and 19.3%, respectively. In the present study, the frequency of *p53* codon 72 genotypes among the controls and patients non- closely conformed to Hardy-Weinberg equilibrium and we found significant differences in the genotype or allele frequencies between the patients and the control groups. Moreover, the results showed increases in the *Arg/Arg* genotype and a decrease in *Pro/Pro* genotype among patients and an increase in the *Arg* allele.

In agreement with prior studies on breast, stomach and colorectal cancer in Iran we found significant differences in the genotype or allele frequencies between the patients and the control groups (Mojtahedi et al., 2010).

A number of studies have failed to demonstrate the association between the *p53* codon 72 polymorphism colorectal cancers. For example, Hamajima et al. (2002) analyzed this SNP in 147 colorectal cancer patients, and 241 non-cancer patients, in a Japanese population, and found no significant association between the genotype or allele frequencies and the diseases

(Hamajima et al., 2002). However, other studies found the *p53* codon 72 polymorphism to be a genetic risk factor for colorectal cancer (Mammano et al., 2009).

The genotype frequencies of *TP53* codon 72 in 180 sporadic colorectal adenocarcinomas and 180 healthy individuals from Isfahan province of Iran in 2008 was showed a significant difference between cases and controls for the *Arg/Arg* genotype compared with (grouped) *Arg/Pro* and *Pro/Pro* genotypes. The *Arg* allele was found more often in patients than in controls. The allele and genotype frequencies of the *p53* codon 72 *Arg*/*Pro* polymorphism in stomach, colorectal adenocarcinoma and breast cancer patients and compared to the healthy individuals from the population of southern Iran showed non significant differences in the genotype or allele frequencies between the patients and the control groups (Khadang et al., 2007). Case and control studies conducted in Japan and Turkey failed to find an association between the prevalence of *p53* polymorphism and colorectal cancer. In this study the allelic frequencies were concordant between the controls and colorectal cancer cases, reaching a frequency of approximately 0.6 for the *Arg* allele.

Conclusion

The findings of the present study indicate that *TP53* codon 72 polymorphism may be a genetic predisposing factor for colorectal adenocarcinomas and *p53Arg72* protein may be correlated with possible increased risk of this kind of cancers in south west of Iran.

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