TP53 codon 72 Polymorphism and P53 Protein Expression in Colorectal Cancer Specimens in Isfahan

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Abstract- The TP53 tumor suppressor gene plays important roles in genomic stability. A common polymorphism at codon 72 of TP53 gene has been associated with increased risk for many human cancers. The p53 protein is expressed in colorectal cancer, but the reported prevalence of its expression varies widely. In the present study, the p53 protein expression in different genotypes of its codon 72 , was investigated. We undertook a case–control study on 250 controls and 250 paraffin block specimens of sporadic colorectal adenocarcinomas from the city of Isfahan. PCR amplification of TP53 codon 72 polymorphism: TP53 codon 72 genotypes were detected by PCR using specific primer pairs for amplifying the proline or the arginine Alleles. The PCR reaction was done separately for each of the two polymorphic variants. The amplified products were subjected to electrophoresis on 1% agarose gel in 1× TBE buffer and visualized on a transilluminator using ethidium bromide. Immunohistochemical Staining: We evaluated the expression patterns of p53 protein, as potential prognostic marker in colorectal cancer specimens by immunohistochemical staining. Statistical analyses**:** The χ_2-test was used to assess the significance of any difference in the prevalence of TP53 codon 72 polymorphism between colorectal cancer patients and controls. The odds ratio and 95% CI (confidence intervals) was used as a measure of the strength of the association. Statistical significance level was set to $P \leq 0.05$. In control samples, the genotype distribution for TP53 polymorphism showed 30.4%, 45.2% and 24.4% for the arginine/arginine, arginine/proline and proline/proline genotypes, respectively. Allelic frequencies corresponded to 0.663 for the arginine allele and 0.338 for the proline allele. In the cancer group 38.8% of the cases were arginine/arginine, 40.4% were arginine/proline and 20.8% were proline/proline. The corresponding frequencies were 0.590 for the arginine allele and 0.410 for the proline allele. A significant difference between cases and controls was found for the arginine/arginine genotype compared with (grouped) arginine/proline and proline/proline genotypes (Odds Ratio = 1.451 (1.002-2.103), P=0.048). Overexpression of p53 was observed in 50.8 percent of cancer specimens which most of them were arginine/arginine genotype (P<0.001). TP53 polymorphism and arginine/arginine genotype may be correlated with overexpression of p53 and increased risk for colorectal cancer in city of Isfahan.

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Introduction

Colorectal carcinogenesis is a complex multistage process that show a high frequency of TP53 alterations and the large majority of these cancers are adenocarcinomas (1,2). TP53 is the most important tumor suppressor gene that is involved in many pathways such as apoptosis, cellular transcriptional regulation, and cell cycle control (3,4). The p53 protein has important role in cell cycle control, being involved in G1-phase arrest for DNA repairs or activation of the cell death machinery (5). Accumulation of the protein in the cytoplasm being done following DNA damage, then the protein translocates to the nucleus and activates gene transcription machinery for cell cycle arrest, to allow repair of damaged DNA (6). Also p53 protein, in response to an excessive DNA damage, would activate programmed cell death pathway, through transcriptional control of several genes (7,8). TP53, located on chromosome 17p13, is one of the most mutated genes

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affecting many types of human cancers (9,10). In addition to mutations, several polymorphisms in the wild-type TP53 gene locus have been detected which could alter its function (11,12). Among the 14 polymorphisms identified in the TP53 gene, the most commonly polymorphism in the general population which associated with cancer development is the codon 72 Arg (arginine) to Pro (proline) substitution (13). The TP53 Arg72Pro, located in exon 4 at codon 72, involving a guanine to cytosine nucleotide exchange, which leads to nonconservative change of an Arg to Pro. Because of functional differences between the two polymorphic variants of TP53, genotype at codon 72 may affects susceptibility to colorectal cancer development. Also it was proposed that the p53 codon 72 polymorphism influences the expression of p53 (14), and it is logical that this polymorphism may play a role in p53 protein expression in colorectal cancer. The p53 protein is expressed in colorectal cancer, but the reported prevalence of its expression varies widely (15-17). So, in the present study, the p53 codon 72 polymorphism and p53 protein expression were characterized for a group of patients from city of Isfahan in order to explore a possible association between colorectal cancer with this polymorphism and to determine if there is a correlation between this polymorphism and p53 protein expression.

Patients and Methods

Study population and samples

We performed a case–control study on 250 paraffin blocks of sporadic colorectal adenocarcinomas and 250 healthy controls, in order to examine possible associations between the Arg72Pro alleles and the risk of cancer. Incident colorectal cancer cases (histologically confirmed) attending the Alzahra Hospital (Isfahan) over the period 2002-2006 made up the case group. Proximal tumors were defined as occurring in the cecum through to the transverse colon; tumors in the splenic flexure, descending and sigmoid colon were defined as being distal. Other disorders of colorectal region such as HNPCC, familial adenomatous polyposis, Inflammatory Bowel Disease (IBD), Hamartoma, simultaneous occurrence of adenomas, previous or synchronous adenocarcinomas were excluded from this study. As control group, we used peripheral blood from 250 healthy age and sex matched persons. Controls were noncancer persons who already underwent colonoscopy.

DNA isolation from colorectal tissue and blood samples

Genomic DNA from the tumors and blood samples was prepared using High pure PCR Template preparation DNA isolation kit (Roche, Germany) for tissue and whole blood, according to manufacturer's instructions.

PCR amplification of TP53 codon 72 polymorphism

The TP53 codon 72 Pro allele were detected by PCR using the primer pair p53Pro+/ p53Pro- (p53Pro+: 5′-GCCAGAGGCTGCTCCCCC; and P53Pro-: 5′-CGTGCAAGTCACAGACTT) and the p53 codon 72 Arg allele by the primer pair p53Arg+/p53Arg- (p53Arg+: 5′-TCCCCCTTGCCGTCCCAA and p53Arg-: 5′-CTGGTGCAGGGGCCACGC) as previously described [13]. Between 100 to 300 nanograms DNA was used as template in a 25 µl PCR reaction mixture containing 1.5 µmol MgCl2, 1 U Taq polymerase (Sinagen, Co. Ltd., Tehran, Iran) and 2 µmol either of the primer pairs.

PCR cycling conditions were carried out with an initial denaturation step for 3 min at 94 ºC, followed by 35 cycles of 30 s at 94 ºC, 30 s at 60ºC (for Arg) or 54 ºC (for Pro) and 30 s at 72 ºC. A final extension step was performed at 72 ºC for 5 min. The PCR reaction was done separately for each of the two polymorphic variants. The amplified products were subjected to electrophoresis on 1% agarose gel in 1× TBE buffer and visualized on a transilluminator using ethidium bromide.

Immunohistochemical staining

Five-micron sections containing tumor tissue and normal colonic mucosa as internal positive control were cut slides. After routine deparaffinization and rehydration including an endogenous peroxide block with methanol-peroxide for 30 minutes, the sections were microwaved for nonenzymatic epitope retrieval at 800W for 5 minutes. Fifty milliliters H_2O were replenished, and an additional microwaving step followed at 800W for 5 minutes. The slides were let cool in the buffer for 20 minutes. The immunostainings were performed by using the avidin-biotin-peroxidase amplification system. After a blocking in normal serum for 20 minutes, the slides were incubated with the primary antibodies overnight at room temperature. The biotinylated secondary antibody was applied for 30 minutes, and is followed by 3 applications of DAB/0.02% hydrogen peroxide. The slides were washed with phosphate-buffered saline (PBS) between the

incubations. A Harris hematoxylin counterstain was used.

Statistical analyses

The χ2-test was used to assess the significance of any difference in the prevalence of TP53 codon 72 polymorphism between colorectal cancer patients and controls. The odds ratio and 95% CI (Confidence Intervals) was used as a measure of the strength of the association. Also associations between qualitative variables were evaluated using the χ^2-test. Statistical significance level was set to $P \leq 0.05$.

Results

This analysis included 250 adenocarcinomas and 250 cancer-free control subjects. The general and clinicopathological characteristics of the cases are shown in table 1. The age of 250 patients (104 women and 146 men) ranged from 32 to 93 years (mean age 65.16±12.34 years in men, 62.14±15.78 years in women).

To analyze the codon 72 polymorphism, we used a PCR-based assay that specifically, amplify either TP53 Pro or TP53 Arg allele and give a PCR product by using specific primers for Pro allele (Figure 1) and/or Arg allele (Figure 2) respectively. Detection of TP53 codon 72 polymorphism by allele specific PCR was successfully conducted in all cases and controls. The distribution of the three different genotypes of codon 72 in exon 4 of TP53 in our cases and controls is shown in table 2. In control samples, the genotype distribution for p53 polymorphism showed 30.4%, 45.2% and 24.4% for the Arg/Arg, Arg/Pro and Pro/Pro genotypes, respectively. Allelic frequencies corresponded to 0.663 for the Arg allele and 0.338 for the Pro allele (Table 3). In the cancer group 38.8% of the cases were Arg/Arg, 40.4% were Arg/Pro and 20.8% were Pro/Pro (Table 2). The corresponding frequencies in this group were 0.590 for the Arg allele and 0.410 for the Pro allele (Table 3). A significant difference between cases and controls was found for the Arg/Arg genotype compared with (grouped) Arg/Pro and Pro/Pro genotypes (Odds Ratio = 1.451 (1.002-2.103), P=0.048).

Table1. General and clinicopathologic data of patients with colorectal Cancer

Factor	N (%)
Sex	
Male	146 (58.4 %)
Female	104 (41.6 %)
Age	
≤ 59	82 (32.8 %)
≥ 60	168 (67.2 %)
Localization	
Proximal	153 (61.2 %)
Distal	97 (38.8 %)
Dukes stage	
A- B	78 (31.2 %)
C-D	172 (68.8 %)
TNM staging	
I	62 (24.8 %)
II	74 (29.6 %)
III	82 (32.8 %)
IV	32 (12.8 %)

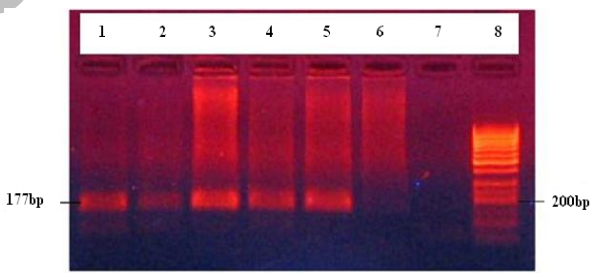

Figure 1. PCR amplification of the TP53 codon 72(electrophoresis in 1% agarose gel) in 6 colorectal adenocarcinoma specimens.
lane 1-5: positive for Pro allele (177bp)
lane 6: negative for Pro allele
lane7: negative control
lane8: DNA marker

Table 2. Distribution of TP53 codon72 polymorphism genotypes among colorectal cancer cases and controls in Isfahan

Genotype	Cases(n=250)		Controls(n=250)		Odds Ratio (95% CI)
	n	%	n	%	
A/A	97	38.8%	76	30.4%	1.451*
A/P	101	40.4%	113	45.2%	(1.002-2.103)
P/P	52	20.8%	61	24.4%	

A/A: Arg/Arg genotype; A/P: Arg/Pro genotype;
P/P: Pro/Pro genotype; n: number
CI : Confidence Intervals * : χ^2 test, P=0.048 (Arg/Arg genotype compared with (grouped) Arg/Pro and Pro/Progenotypes)

Table 3. Allelic frequencies of TP53 codon 72 among colorectal cancer cases and controls in Isfahan

Allele	Patients	Controls	Odds Ratio (95% CI)
Arg	0.590	0.663	0.733*
Pro	0.410	0.338	(0.558-0.964)

CI: Confidence Intervals

* : $\chi 2$ test, P=0.026

Table 4. P53 Expression in colorectal cancer specimens

Genotypes of tumor specimens	N	P53 Expression	
		Positive n(%)	Negative n(%)
A/A	97	69 (71.1)	28 (28.9)
A/P	101	48 (47.5)	53 (52.5)
P/P	52	10 (19.2)	42 (80.8)
Total	250	127 (50.8)	123 (49.2)
		P <0.001	

A/A: Arg/Arg genotype;
A/P: Arg/Pro genotype;
P/P: Pro/Pro genotype;
n: number

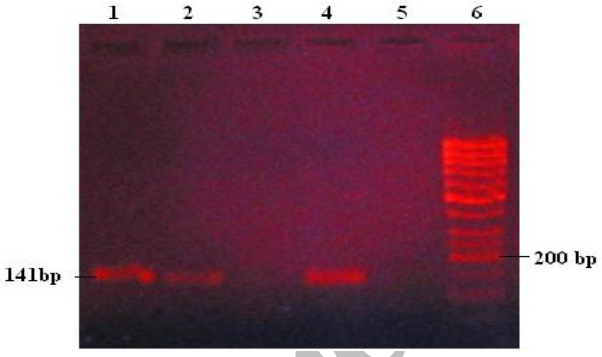

Figure 2. PCR amplification of the TP53 codon 72(electrophoresis in 1% agarose gel) in 6 colorectal adenocarcinoma specimens.
lane 1,2, 4 : positive for Arg allele (141bp)
lane 3: negative for Arg allele
lane5: negative control
lane 6: DNA marker

The Arg allele was found more often in patients than in controls (Odds Ratio = 0.733 (0.558-0.964), P=0.026). Overexpression of P53 was seen in 50.8 percent of colorectal specimens and the most of them were Arginine/Arginine genotype (P<0.001) (Table 4).

Discussion

Polymorphism in p53 codon 72 produces two different p53 proteins because of a single base change altering CGC to CCC in the fourth exon of the p53 gene, altering amino acid residue 72 from Arg to Pro. Of the Arg/Arg, Arg/Pro and Pro/Pro genotypes, Arg/Arg induces apoptosis with faster kinetics and suppresses transformation more efficiently than Pro/Pro (18,19). An association of the TP53 codon 72 polymorphism with several cancers susceptibilities has been reported (20-30). In particular, both Arg and Pro alleles have been shown to be associated with a high risk of malignancy. The role of the Arg/Pro polymorphism in colorectal cancer susceptibility was examined in a few studies (31-42), which reported controversial results. We investigated the genotype frequencies of TP53 codon 72 in 250 sporadic colorectal adenocarcinomas and 250 healthy individuals from Isfahan. We found a significant difference between cases and controls for the Arg/Arg genotype compared with (grouped) Arg/Pro and Pro/Pro genotypes (Odds Ratio = 1.451 (1.002-2.103), P=0.048). The Arg allele was found more often in patients than in controls (Odds Ratio = 0.733 (0.558-0.964), P=0.026). These findings are in agreement with the original study of Storey *et al*. on cervical cancer (43). They showed that p53Arg72 protein is more susceptible to degradation by the HPV E6 proteins, and degradation of p53 protein by HPV E6 is correlated with increased risk for HPV-associated cancers. In this study we did not consider HPV infections in accordance to detection of p53 genotypes and it is an important issue for future studies. Our finding also seems to be consistent with the results reported by Perez *et al*. (31) which support an appreciable association between the Arg allele and colorectal cancer. However there are contradictory findings about the mechanisms which lead to the increase of the Arg allele in human cancers (44-46) implicate that the involvement of TP53 polymorphism in human cancer demands further study.

Although the exact effect of the p53 codon 72 polymorphism on the function of p53 protein remains unknown, it is proposed that this polymorphism

influences the expression of the p53 gene (14). As such, we suspected that the p53 polymorphism may play a role in p53 protein expression in colorectal cancer. The p53 overexpression detected by immunohistochemistry is based on the accumulation of p53 protein in cells. In colorectal carcinomas the correlation between p53 gene status and p53 immunostaining was estimated in over 70% of the cases (47). The p53 overexpression was detected in our study in 127 cases (50.8%) which most of them were Arg/Arg genotype (P<0.001).So we demonstrated that p53 overexpression was associated with the p53 codon 72 polymorphism and tended to be more frequent in the colorectal carcinomas with a Arg/Arg genotype. In previously reported studies, p53 overexpression was observed in 60.6% (15); 67.3% (16) and30% of the cases (17).

In conclusion the findings of the present study indicated that TP53 codon 72 polymorphism may be a genetic predisposing factor for colorectal adenocarcinomas and p53Arg72 protein may be correlated with p53 overexpression and increased risk for colorectal cancer in Isfahan.

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