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Original Article

Analysis of TP53 Codon 72 Polymorphism in Mucinous and Non-Mucinous Colorectal Adenocarcinoma in Isfahan, Iran

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Abstract

Background: The tumor suppressor gene TP53 (alias p53) located on chromosome 17 is involved in various cancers. Case-control studies have shown that p53 codon 72 polymorphism modulates the prognosis and susceptibility to various malignancies. We undertook the present study to explore a possible association between mucinous and non-mucinous adenocarcinomas with different genotypes or alleles at codon 72 of TP53.

Methods: The genotype distribution and allelic frequencies for p53 polymorphism was assessed in 46 and 134 specimens from patients with colorectal mucinous and non-mucinous adenocarcinomas, respectively, by using allele-specific PCR.

Results: The PCR products were 177bp for proline allele and 141bp for arginine allele. In the mucinous samples, the genotype distribution for p53 polymorphism showed 63%, 23.9%, and 13.1% for the Arg/Arg, Arg/Pro, and Pro/Pro genotypes, respectively. In the non-mucinous specimens 32.1% of the cases were Arg/Arg, 48.5% Arg/Pro, and 19.4% pro/pro. A significant difference between the two types of adenocarcinomas for the Arg 72 Arg genotype compared with (grouped) Arg 72 Arg and Pro 72 Pro genotypes was noted [OR=3.61 (1.76-7.27), P<0.001]. The arginine allele was found more often in patients with mucinous adenocarcinoma [OR=1.85 (1.07-3.19), P<0.03]. A higher portion of Dukes stage C was noted in the mucinous specimens (P<0.02) and also mucinous specimens were seen more often at advanced TNM stages (P=0.01).

Conclusion: The Arg/Arg genotype at p53 codon 72 is more prevalent in mucinous colorectal carcinoma and the arginine allele may contribute to mucinous carcinogenesis. The proline allele was associated with higher Duke's staging in non-mucinous adenocarcinoma.

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Keywords ● TP53 ● polymorphism ● colorectal neoplasm● mucinous ● adenocarcinoma

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Introduction

Colorectal cancers have a main diagnostic specification that is the degree to which they generate and secrete mucin. Mucinous colorectal adenocarcinomas make

10-15% of colorectal carcinomas with a distinct set of clinical, pathological, and molecular genetic features;[1-5] however, most of colon cancers are non-mucinous colorectal adenocarcinomas.

P53 protein has important role in cell cycle control, being involved in G_1-phase arrest for DNA repairs or activation of the cell death machinery.[5] TP53 gene, located on chromosome 17p13, is one of the most mutated genes that affects many types of human cancers.[6,7] In addition to mutations, several polymorphisms in the wild-type p53 gene locus have been detected, which could alter its function.[8,9] Among the 14 polymorphisms identified in the TP53 gene, the most commonly polymorphism in the general population that is associated with cancer development is the codon 72 arginine (Arg) to proline (Pro) substitution.[10] These variants may be associated with tumor susceptibility because they interfere with the ability of TP53 to activate apoptosis, and might account for ethnic variation in cancer frequency.

The role of codon 72 polymorphism of p53 gene had been noted in patients with colorectal cancer in a few studies,[11-18] but the association between this polymorphism and mucinous or non-mucinous type of the disease development had not been determined. Because of functional differences between the two polymorphic variants of p53 that could alter its function, we hypothesized that genotype at codon 72 may affect the susceptibility to development of mucinous type. We undertook the present study to explore a possible association between these two types of colorectal adenocarcinoma and different genotypes or allels at codon 72 of TP53 tumor suppressor gene.

Patients and Methods

Study Population and Samples

One hundred eighty paraffin-embedded blocks of colorectal adenocarcinomas were collected randomly from patients proven to have colorectal carcinomas who underwent tumor resection from January 2002 to December 2006 at the Surgery Ward, Alzahra Hospital, affiliated to Isfahan University of Medical Sciences (Isfahan, Iran). There were 46 specimens of mucinous and 134 specimens of non-mucinous type. The patients' data including age, gender, family history of colorectal cancer, and clinicopathological presentation of these patients extracted from their charts. The site and maximum length of the tumors were also recorded.

DNA Isolation from Colorectal Tissue Samples

Genomic DNA from the tumor samples was prepared using high pure PCR template preparation DNA isolation kit (Roche, USA), according to manufacturer's instructions. Briefly 10-micron sections were cut from formalin fixed, paraffin-embedded blocks of each tumor. These sections were deparaffinized in Eppendorf tubes (2×1 ml xylene for 10 minutes each, and 2×1 ml 100% ethanol for 10 minutes each). After air drying at room temperature, samples were suspended in 1 ml DNA extraction buffer (0.3 mg/ml proteinase K, 100 mmol/L NaCl, 10 mmol/L Tris-HCl pH 8.0, 25 mmol/L EDTA pH 8.0, and 0.5% sodium dodecyl sulfate) and were incubated with shaking at 55˚C overnight. Additional proteinase K was added 24 hours and 48 hours later for a total incubation time of 72 hours. A 500 µl sample mixed with 500µl phenol chloroform isoamyl was incubated at room temperature for 10 minutes and centrifuged. DNA in the top layer was collected and precipitated with 250 µl of 7.5 mol/L ammonium acetate and 1 mL of ice-cold 100% ethanol. The DNA was pelleted by centrifugation (14,000 rpm for 20 minutes). DNA was dissolved overnight in 20-40 µl of Tris-EDATA buffer (10 mmol/L Tris, 1 mmol/L EDTA).

PCR Amplification of p53 Codon 72 Polymorphism

The p53 codon 72 proline allele were detected by PCR using the primer pair p53Pro+/p53Pro- (p53Pro+: 5′-GCCAGAGGCTGCTCCCCC; and P53Pro-: 5′-CGTGCAAGTCACAGACTT) and the p53 codon 72 arginine allele by the primer pair p53Arg+/p53Arg- (p53Arg+: 5′-TCCCCCTTGCCGTCCCAA and p53Arg-: 5′-CTGGTGCAGGGGCCACGC) as previously described.[19] Between 100 and 300 nanograms DNA was used as template in a 25µl PCR reaction mixture containing 1.5µmol mgcl2, 1U Taq polymerase (Sinagen, Iran) and 0.2µmol either of the primer pairs.

PCR cycling conditions were carried out with an initial denaturation step for 3 min at 94ºC, followed by 35 cycles of 30s at 94ºC, 30s at 60˚C (for Arg) or 54ºC (for Pro) and 30s at 72˚C. A final extension step was performed at 72˚C for 5 min. The PCR reaction was performed separately for each of the two polymorphic variants. The amplified products were subjected to electrophoresis on 1% agarose gel in 1× Tris-Borate-EDTA buffer and visualized on a transilluminator using ethidium bromide. The resulting PCR products were 177bp for Pro allele and 141bp for Arg allele.

Statistical Analyses

Comparisons of p53 polymorphism and

clinicopathological characteristics between mucinous and non-mucinous types of colorectal adenocarcinomas and comparison of allelic frequencies for p53 polymorphism between different Dukes staging in each group were performed using Chi square, Fisher's exact, and Student's *t* tests respectively. Depending on the size of the population, Fisher's exact test or Chi square test was used for categorical variables. Chi square test was used when the number of subjects in each category was at least five. Otherwise, Fisher's exact test was used. Student's *t* test was used for continuous variables. The odds ratio and 95% confidence intervals (CI) were used as measure of the strength of the association. Statistical significance level was set to P≤0.05. All statistical tests were performed using SPSS version 11.5.

Results

The age of 180 patients (77 women and 103 men) ranged from 32 to 91 years. Thirty patients (16.6%) had a positive family history of colorectal cancer. The general and clinicopathological characteristics of the patients are shown in table 1.

The mucinous adenocarcinomas were found more common in the proximal portion of the large intestine (cecum, ascending and transverse colon; P=0.03). In particular, These tumors were most commonly seen in the transverse colon (table 1). The mean dimensions of such tumors were larger significantly (7.6±2.04cm *vs* 4.8±2.23cm; P=0.04). Also, a higher portion of Dukes stage C was noted in mucinous type (P<0.02) and the tumors were seen more often at advanced TNM stages (P=0.01). Rectal mucinous adenocarcinomas were found more often in female patients who often were diagnosed at advanced stages.

For analyzing the codon 72 polymorphism, we used a PCR-based assay that specifically amplify either p53 Pro or p53 arginine allele using specific primers for proline and arginine alleles separately. The resulting PCR products were 177bp for proline allele and 141bp for arginine allele (figure 1). Detection of TP53 codon 72 polymorphism by allele specific PCR was successfully conducted in all cases. The distribution of the three different genotypes of codon 72 in exon 4 of p53 is shown in table 2.

Table 1: Clinicopathological characteristics of the colorectal cancer specimens

Parameters	MCA	NMCA	P value
Number	46	134	
Male/Female ratio	27/19	76/58	0.81
Mean age (yr)	61.3±12.3	64.6±13.1	0.74
Mean tumor length (cm)	7.6±2.04	4.8±2.23	0.04
Location			
Cecum	8	13	0.03
Ascending colon	2	12	
Transverse colon	15	19	
Descending colon	4	10	
Sigmoid colon	8	32	
Rectum	9	48	
Duke's stage			
A	1	14	0.01
B	2	26	
C	36	83	
D	7	11	
Positive lymph node metastases	25	55	0.12
Positive distant metastases	5	12	0.36
TNM staging			
I	4	41	0.01
II	19	38	
III	17	48	
IV	6	7	

MCA: Mucinous colorectal adenocarcinoma, NMCA: Non-mucinous colorectal adenocarcinoma

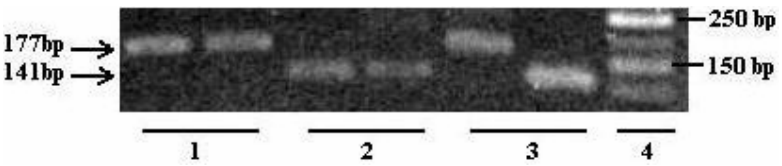

Figure 1: Analysis of p53 codon 72 polymorphism in three patients with colorectal adenocarcinoma. The amplification of the arginine allele with specific primer pair gives a PCR product of 141 bp. The other specific primer pair amplifies a 177-bp fragment corresponding to the proline allele. Case 1: Pro/Pro, case 2: Arg/Arg, case 3: Arg/Pro, and 4: DNA marker.

Table 2: Distribution of P53 codon 72 polymorphism genotypes among mucinous and non-mucinous specimens

Genotype	MCA (N=46) N(%)	NMCA (N=134) N(%)	OR (95% CI)	P value
A/A	29(63.0)	43(32.1)	3.61 (1.76-7.27)	P<0.001
A/P+ P/P	17(37)	91(67.9)		

N: Number, A/A: Arg/Arg genotype, A/P: Arg/Pro genotype, P/P: Pro/Pro genotype, OR: Odds ratio, MCA: Mucinous colorectal adenocarcinoma, NMCA: Non-mucinous colorectal adenocarcinoma

In the mucinous samples, the genotype distribution for p53 polymorphism revealed 63%, 23.9%, and 13.1% for the Arg/Arg, Arg/Pro, and Pro/Pro genotypes, respectively. Allelic frequencies corresponded to 0.749 for the arginine allele and 0.251 for the proline allele (table 3). In non-mucinous specimens, 32.1% of the cases were Arg/Arg, 48.5% were Arg/Pro and 19.4% were Pro/Pro. The corresponding frequencies were 0.563 for the arginine allele and 0.437 for the proline allele. A significant difference between mucinous and non-mucinous types was found for the Arg/Arg genotype compared with grouped Arg/Pro and Pro/Pro genotypes [OR=3.61 (1.76-7.27), P<0.001]. The arginine allele was found more prevalent in patients with mucinous type [OR=1.85 (1.07-3.19), P<0.03]. Also the proline allele was found more prevalent in non-mucinous specimens with advanced stages [OR=2.93 (1.65-5.20), P<0.001] (table 4). In mucinous specimens, there was no association between distribution of the two alleles of codon 72 and Dukes Classification (table 4).

Discussion

We investigated the genotype frequencies of p53 codon 72 in 46 mucinous and 134 non-mucinous specimens of colorectal adenocarcinomas in Isfahan; central area of Iran. Similar to the findings of other studies,[1,20] we found that mucinous type of the disease were more common in proximal colon (cecum, ascending and transverse colon) and especially in the transverse colon. The size of the mucinous tumors was larger than non-mucinous ones. Patients with mucinous type were at a similar age with the patients with non-mucinous type.

Our results were consistent with other studies that showed patients with mucinous adenocarcinoma present at more advanced stage of disease than those with other types of carcinoma.[1] Mucinous adenocarcinoma with microsatellite instability, a phenomenon that reflects accumulated errors in DNA microsatellite repeat sequences following inactivation of a DNA mismatch repair (MMR) enzyme, often arise in the setting of hereditary non-polyposis colorectal cancer.[20] Loss of MMR proteins apparently is an important event in the process of carcinogenesis or in a primary step of tumor progression in hereditary non-polyposis colorectal cancer.[20] In the present study, hereditary factor was pointed to be common in mucinous adenocarcinoma; 30 specimens (16.6%) had a positive family history of colorectal cancer.

We found a significant difference between the two types of colorectal cancer for the Arg/Arg genotype compared with grouped Arg/Pro and Pro/Pro genotypes. Mucinous

Table 3: Allelic frequencies of TP53 codon 72 among mucinous and non-mucinous adenocarcinomas

Specimens	Arg	Pro	OR (95% CI)	P value
MCA	0.749	0.251	1.85 (1.07-3.19)	0.025
NMCA	0.56	0.43		

Arg: Arginine, Pro: Proline, OR: Odds ratio, MCA: Mucinous colorectal adenocarcinoma, NMCA: Non-mucinous colorectal adenocarcinoma

Table 4: Allelic frequencies of Tp53 codon 72 among MCA and NMCA specimens with different Dukes staging

Allele / Specimens with different Dukes stages	Arg N(%)	Pro N(%)	OR (95% CI)	P value
NMCA				
A-B	59(73.8)	21(26.3)	2.93 (1.65-5.20)	P<0.001
C-D	92(48.9)	96(51.1)		
MCA				
A-B	5(83.3)	1(16.7)	1.71 (0.19-15.42)	1.00
C-D	64(74.4)	22(25.6)		

Arg: Arginine, Pro: Proline, OR: Odds ratio, NMCA: Non-mucinous colorectal adenocarcinoma, MCA: Mucinous colorectal adenocarcinoma

adenocarcinoma specimens showed higher frequency of Arg/Arg. Also the arginine allele was found more prevalent in patients with mucinous adenocarcinoma. Our finding seems to concur with the results reported by Perez and co-workers,[11] who reported an appreciable association between the arginine allele and colorectal cancer.

In addition, we found that the proline allele was more frequent in non-mucinous adenocarcinoma specimens with advanced stages. However, in mucinous specimens there was no association between the distribution of the two alleles of codon 72 and Dukes classification.

Our results in non-mucinous adenocarcinoma specimens were consistent with previous study by Lung and colleagues who showed that patients with CCC polymorphism (Pro allele) presented at a more advanced stage of disease than those with CGC polymorphism (Arg allele).[12] However, our findings are not consistent with their findings in patients with mucinous adenocarcinoma.

In our study the Pro allele was accompanied by higher Duke's staging in non-mucinous specimens. In the study by Koushik and colleagues no significant association between p53 Arg72Pro genotype and colorectal cancer was shown. However, they suggested that Arg72Pro might play a role in the early stages of colorectal cancer and possibly in progression to invasive disease.[18]

The discriminatory retention of the arginine allele in the cancerous tissue has been traced in several tumors, such as urinary system, vulval, head and neck, esophageal, lung, and breast cancer.[21-25] The arginine allele is established to be more sensitized to degradation by human papillomavirus E6 protein than the Pro allele.[19] Deletion of a codon 72 region has been demonstrated to damage the ability of p53 to inhibit neoplastic cell growth in culture and ensues in loss of apoptotic work.[26] It antecedently was described that p53 mutant protein overexpression was importantly heightened by the presence of Arg 72 allele in the esophageal tumor.[27] It has also been exhibited in a set of squamous cell carcinomas arising in Arg/Pro germline heterozygotes, that loss of heterozygosity preferred loss of the Pro allele, declaring that the Arg polymorphic residue may alter mutational models.[28] In the present study, because of no access to blood samples or normal tissues of patients, we did not check the phenomenon of loss of heterozygosity.

Analogous veer was established in the association of Arg/Arg genotype with p53 mutation. Moreover, it has been shown that mutated p53 protein can attach and alter its homolog, p73, thus nullifying p73-induced apoptosis .[29] This activity was observed to be substantially intensified when the p53 codon 72 encoded Arg. Nevertheless, p73 up-regulation in colorectal cancer has been associated with tumor progression and aggressiveness.[30,31] Because of antonymous results on the role of Arg allele in human cancers, the involvement of p53 polymorphism in human cancer claims further research work. In the present study, because of the limited sample availability and their poor quality, neither p53 mutation analysis nor p73 staining were done. Assessment of the interplay between p53 alterations and p73 function relevant to p53 codon 72 polymorphism in mucinous specimens needs additional studies.

While extra genetic factors, as well as environmental and lifestyle considerations, distinctly need to be taken into account, our results render evidence that the codon 72 Arg allele may contribute to the mucinous carcinogenesis. Deregulation of differentiation and renewal of goblet cells, which is the major cell type accountable for secretion of mucins, may be affected by the effect of the Arg allele at p53 codon 72 on functioning of the p53 protein and this phenomenon probably have a critical effect on colon cancer progression. It is suggested that substitution of a Pro by Arg at this residue may contribute partly to decline of apoptosis and a possible decrease in vindication against carcinogenesis. These findings are consistent with the hypothesis that alterations of the proline-rich region of p53 may change the potency of the protein to modulate processes in the cell involved in apoptosis or cell-cycle arrest. Because the development and progression of mucinous adenocarcinoma is a dynamic process of cell proliferation and cell degradation, it might be a disadvantage if cells react with a lower extent of physiologically necessary cell-cycle arrest in the p53 Arg allele carriers compared with the Pro carriers. The lower amount of cell-cycle arrest in these carriers might lead to genetically misguided cell growth. In the light of recent studies on the effect of the Arg allele at p53 codon 72 on functioning of the p53 protein, such as the capability to evoke apoptosis, to place to the mitochondria, to be degraded by the proteasome, and to regulate gene transcription, our findings justify further investigations.

Genetic heterogeneity and environmental factors should be considered in the pathogenesis of colorectal cancer. It is also possible that P53 codon 72 polymorphism could be in linkage disequilibrium with other acknowledged

etiological variants,[32] which would probably differ in different ethnic populations. The present study was not controlled for these potential predisposing factors.

In conclusion, our results indicated Arg/Arg genotype at p53 codon 72 is more prevalent in mucinous colorectal carcinoma and the Arg allele may contribute to mucinous carcinogenesis. Pro allele was associated with higher Duke's staging in non-mucinous adenocarcinoma.

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Conflict of Interest: None declared

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