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Characterization and Prognostic Value of Mutations in Exons 5 and 6 of the p53 Gene in Patients with Colorectal Cancers in Central Iran

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Background/Aims: We aimed to investigate the relationships among various mutations of the p53 gene and their protein products, histological characteristics, and disease prognosis of primary colorectal cancer in Isfahan, central Iran. **Methods:** Sixty-one patients with colorectal adenocarcinoma were enrolled in the study. Mutations of the p53 gene were detected by single-stranded conformation polymorphism and DNA sequencing. The protein stability was evaluated by immunohistochemistry. Patients were followed up to 48 months. **Results:** Twenty-one point mutations in exons 5 and 6 were detected in the tumor specimens of 14 patients (23%). Of those, 81% and 9.5% were missense and nonsense mutations, respectively. There were also two novel mutations in the intronic region between exons 5 and 6. In 11 mutated specimens, protein stability and protein accumulation were identified. There was a relationship between the type of mutation and protein accumulation in exons 5 and 6 of the p53 gene. The presence of the mutation was associated with an advanced stage of cancer (trend, $p<0.009$). Patients with mutated p53 genes had significantly lower survival rates than those with wild type p53 genes ($p<0.01$). **Conclusions:** Mutations in exons 5 and 6 of the p53 gene are common genetic alterations in colorectal adenocarcinoma in central Iran and are associated with a poor prognosis of the disease. **(Gut Liver 2013;7:295-302)**

Key Words: Colorectal neoplasms; p53 gene mutation; Missense; Nonsense

INTRODUCTION

According to a recent estimate, colorectal cancer is the third most common cancer and the fourth most common cause of cancer deaths worldwide, with 1.2 million incident cases and 609,000 estimated deaths.[1] While incidence and mortality rates of colorectal cancer are decreasing in the North America and many other Western countries, they are increasing in developing and economically transitioning countries.[2] The prevalence of colorectal cancer is increasing in Asia.[3] Currently, it is the fourth most common cancer in Iran.[4]

As a multifactorial malignancy, development and progression of colorectal cancer has been shown to be related to both environmental and genetic factors including mutations in p53 gene.[5-8] The p53 gene, as the most important tumor suppressor gene, is located on the short arm of chromosome 17 p13 which encodes a 35 KD nuclear phosphoprotein.[9,10] The product of normal p53 gene has a role in inhibiting cell proliferation of damaged cell by arresting the cell cycle or inducing them to apoptosis.[11,12] Mutations in the p53 gene prevented cell death in the DNA damaged cells which would be a reason for the resistance to chemotherapy.[13,14] The p53 gene also acts as a transcription factor to regulate expression of more than 100 different genes.[15]

The estimated frequency of p53 mutations in colorectal adenocarcinoma ranges from 25% to 100% in different study populations.[16-20] The wide variation of p53 prevalence can be explained by variations in study population, gender, and detection techniques. The type and location of point mutation is another source of variation. In colorectal cancer, exons 5 and 6 are of special interest of scientists because more than 90% of point mutations of p53 gene occur in theses exons.[21] Individual frequency of p53 mutations in exons 5 and 6 is not clear. While observations in most of the previous studies have suggested relatively high rates of mutation,[22,23] Pan *et al*.[24] reported only one mutation in exons 5 and 6. To date, the lowest and the highest rate of these mutations in patients with colorectal cancer have been reported from Taiwan with 31% and United Kingdom with

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70% respectively.[25] Therefore, this issue is controversial and merits to be investigated.

It is thought that determination of different types of mutations is an important factor for therapeutic propose, i.e., planning chemotherapy or radiotherapy,[26,27] particularly when it is known that some mutations may not induce protein production. The present study was designed to detect and characterize different mutations in exons 5 and 6 of p53 gene in a population of colorectal cancer patients in Isfahan, central Iran. We also tried to assess the relationship between different types of mutations and protein stability in patients with colorectal cancer.

MATERIALS AND METHODS

1. Patients

The present study was conducted on 61 patients with primary intramucosal and invasive colorectal adenocarcinoma. Forty-six (75.4%) samples were from male and 15 (24.6%) were from female patients. These specimens were collected from patients admitted to surgical departments of selected hospitals, mainly Al-Zahra hospital, Isfahan, Iran, between April 2006 and October 2009. A written informed consent was obtained from each patient and tissue samples have been used in the study anonymously. The study protocol was reviewed and approved by ethics committee of Isfahan University of Medical Sciences.

2. Preparations of samples

Sampling was done before receiving radiotherapy and/or chemotherapy. Samples were fixed in formalin 10% immediately after surgical resection, and formalin solution was changed 3 hours later. Tissue passage was done after 24 hours by the tissue processing instrument (Leica, Wetzlar, Germany). Series of 4 μm thick sections from all paraffin blocks were prepared by a rotating microtome. Standard hematoxylin and eosin staining process were applied to all slides. Normal tissue biopsies from nontumoral area of colon were taken from the same patients as control. The modified Dukes' system was used for staging cancer in all patients as described by Zhang *et al*.[28]

3. Immunohistochemistry (IHC)

IHC was carried out as described previously by Leahy *et al*.[29] Briefly, after cutting 4 μm thick sections, all were deparaffinised at 66°C and rehydrated through graded ethanol to water dilutions. For antigen retrieval, sections were placed in buffer citrate solutions (pH 6, 0.01 M) and were then kept at 95°C for 20 minutes. The peroxidase activity of sections was inhibited by 3% H_2O_2 for 5 minutes and washed by phosphate buffer saline for three times. The mouse anti-p53 monoclonal antibody (DAKO, Heverlee, Belgium) was added for 1 hour at 1/50 ratio dilution and washed three times and incubated with biotinylated secondary antibody (DAKO) for 15 minutes at room temperature and washed three times. Streptavidin peroxidise was then added at 20 μL to each slide. The sections were then stained with 3, 3'-diamino benzidine as substrate which produces brown non-soluble precipitation to visualise p53 protein containing cells. Haematoxylin was used to stain the backgrounds. The dehydrated slides then mounted. All slides were blindly reviewed using light microscopy in 10 randomly selected optic fields. The motic light microscope and advanced motic Plus2 software (Ted Pella Inc., Redding, CA, USA) with ×400 were used to count cells. Based on percentage of cells, the slides were scored as negative, weak, average, and strong. The sections with nuclear staining in less than 10% of tumor cells were considered negative (-), 10% to 30% were considered one positive (+), 31% to 50% were considered two positive (++), and staining of over 50% as three positive (+++) (Fig. 1).[23]

4. DNA extraction

DNA of each sample was extracted from 10 μm thick sections of paraffin-embedded tumor tissues. Three slices from each block were placed in 2 mL sterile Eppendorff tubes. The microtome blade carefully cleaned with xylene after preparation of each block. DNA was extracted by the phenol chloroform isoamil alcohol.[30] The same procedure was done for control samples as well.

5. The p53 gene amplification

Exons 5 to 6 in the p53 gene were amplified by polymerase chain reaction (PCR) using following primers. 5´TGTTCACTTGTGCCCTGACT 3´, 5´GGAGGGCCACTGACAACCA 3´, and the length of exons 5 and 6 designed to have 489 bp.

The thermocycler for amplifying exons was set at 94°C for 5 minutes for primary denaturation. A total of 35 cycles were performed including following steps: 1) denaturation at 94°C for 0.5 minutes, 2) annealing at 56°C for 1 minute, 3) extension at 72°C for 1 minute, and 4) final extension at 7°C for 10 minutes. Electrophoresis was performed for all PCR products using 1.5% agarose gel to determine the length of the PCR product compared with a 100 bp standard ladder.[24]

6. Single-stranded conformation polymorphism (SSCP) analysis

SSCP analysis was performed as described previously.[31] Briefly, 6 to 12 μL of the PCR product was added to the same volume of denaturing solution containing 800 μL formamide, 100 μL 1% bromophenol blue, 100 μL 1% xylene cyanol, 3 μL 0.5 M ethylenediaminetetraacetic acid (EDTA), and 1 μL 10 M NaOH per mL solution. Samples were denaturized at 95°C for 5 minutes and immediately transferred to ice to prevent denaturation of DNA. Each sample was loaded into a 10% polyacrylamid gel of 0.5 mm thickness. Electrophoresis was performed in 1×tris borate EDTA at 4°C and at 21 mA over night. Each PCR product was run for electrophoresis at least two times to increase precision and to prevent false positive results. The bands

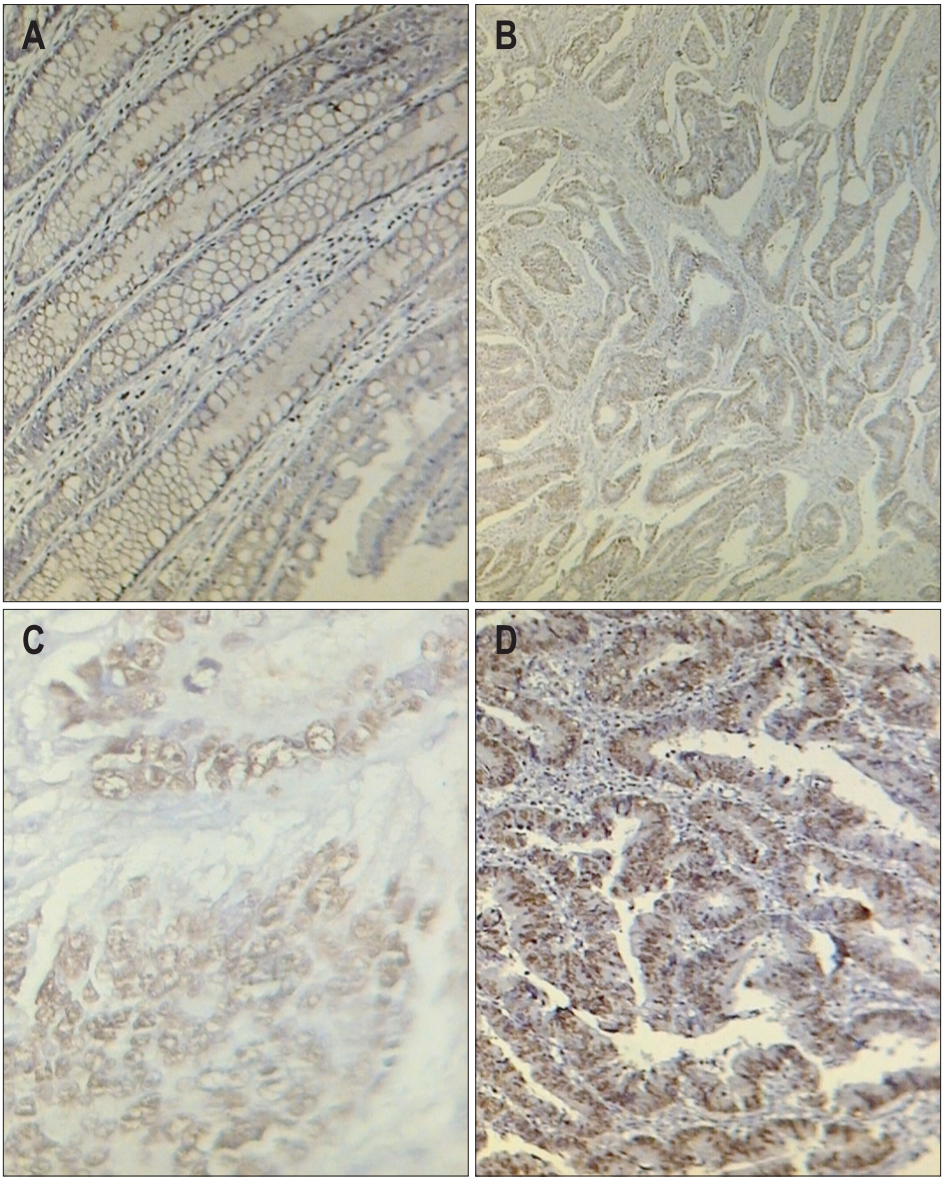

Fig. 1. Immunohistochemistry images of p53 gene products. The specific monoclonal antibody was used at 1/100 dilution. p53 protein stability scored between 1 and 3 in mutated sections. (A) Section of the normal proximal colon (×400). (B) Section of the cancerous mucosa of the proximal colon (×100). (C) Section of the cancerous mucosa of the distal colon (×400). (D) Section of the cancerous mucosa of the distal colon (×100).

were visualized after silver staining of gel.

7. Sequencing

After detection of mutations in exon 5 and 6 of p53 gene, PCR products were analyzed with SSCP. All samples with abnormal migration band were separated and DNA was extracted by a standard kit (Farayand Danesh, Tehran, Iran) and amplified again. These amplified DNA samples were sequenced by Macro-Gen, Seoul, Korea.

8. Follow-up

In order to evaluate relationship between patients' survival and characteristics of p53 mutations, all subjects were followed up to a maximum of 48 months. We contacted family of all patients to ask and check the current status of the relevant patients with colorectal cancer at proper intervals.

9. Statistics analyses

The Pearson's chi-square and Fisher exact tests (if required) were used to explore associations between mutations and histological parameters. Using Kaplan-Meier method, survival of patients with and without mutations and groups in higher and lower cancer stages was explored and differences were tested by Mantel-Cox log-rank test. Results were considered to be significant when p-value was <0.05.

RESULTS

Tumor specimens from 61 patients with colorectal cancer were included in the study for the final analysis of p53 mutations. Tissue specimens of six patients were excluded because of poor DNA quality and six new cases with colorectal cancer were substituted. There were 46 (75.4%) males and 15 (24.6%)

Table 1. Comparison of the Demographic and Histological Features of Colorectal Cancer Patients with and without p53 Mutations

Variable	Mutated p53	Wild type p53	p-value
Gender			0.483*
Male	12 (85.7)	34 (72.3)	
Female	2 (14.3)	13 (27.7)	
Stage of cancer			0.009†
A	2 (14.3)	24 (51.1)	
B	7 (50.0)	17 (36.2)	
C	4 (28.6)	5 (10.6)	
D	1 (7.1)	1 (2.1)	
Tumor differentiation			0.444‡
Well	9 (64.3)	27 (57.4)	
Moderate	3 (21.4)	17 (36.2)	
Poor	2 (14.3)	3 (6.4)	
Tumor location			0.353*
Proximal	3 (21.4)	17 (36.2)	
Distal colon	11 (78.6)	30 (63.8)	

Data are presented as number (%).
*Fisher exact test; †p for trend; ‡Pearson's chi-square test.

females. The higher number of male patients in our study may reflects male predominance of colorectal cancer in the population,[32] although our sample was not necessarily representative of the general population. The age of patients ranged from 44 to 91 years old with the median of 62 (interquartile range, 13). A shown in the Table 1, men and women had similar age at the time of cancer diagnosis (62.5 years vs 60.0 years in male vs female, respectively; Mann-Whitney U test, not significant). The tumor stages, degree of differentiation, and location of tumor in colon and rectum were shown in Table 1. Fourteen patients (23%) had mutations in exons 5 and 6 of which three cases had more than one mutation in their tumoral tissues. There was a male predominance in the rate of p53 mutations (12/46 [26%] vs 2/15 [13%], in males vs females, respectively), but it was not statistically significant (Table 1). In total 21 point mutations were observed in exons 5 and 6 (Table 2). Of those mutations, there were 17 (81%) missenses and two (9.5%) nonsense mutations. There were also two mutations in intronic region between exons 5 and 6, of which one mutation was associated with intron deletion adjacent to exon 6. This deletion caused frameshift mutation in exon 6 which being reported for the first time without any protein production (Table 2).

We examined the histological characteristics of tumors in patients with mutated p53. Except for two patients (14%) who had

Table 2. The Features of p53 Gene Mutations in Exons 5 and 6, Their Protein Products and Associations with Age, Gender, and Tumor Location

Case no.	Gender	Age	Mutation	Amino acid change	Mutation category	Tumor location	Previous reports
1	Male	77	GAT:TAA	Asp:Stop	Nonsense	Proximal	Yes
4	Male	58	GGA:AGG	Gly:Arg	Missense	Proximal	Yes
4	Male	58	AAA:AAT	Lys:Asn	Missense	Proximal	Yes
4	Male	58	ATA:AAT	Ile:Asn	Missense	Proximal	Yes
4	Male	58	GCT:GTC	Ala:Va	Missense	Proximal	Yes
7	Male	63	TCA:TTC	Ser:Phe	Missense	Distal	Yes
9	Male	57	GCA:GGA	Ala:Gly	Missense	Distal	Yes
17	Female	62	TTT:TTA	Phe:Leu	Missense	Proximal	Yes
21	Male	55	CCC:TCC	Pro:Ser	Missense	Distal	Yes
27	Male	68	GGG:GCG	Gly:Ala	Missense	Distal	Yes
27	Male	68	CCG:CTC	Pro:Leu	Missense	Distal	Yes
36	Male	67	CAG:CAC	Glu:His	Missense	Distal	Yes
40	Male	76	ACC:ATC	Thr:Ile	Missense	Distal	Yes
50	Female	69	GAT:GTG	Asp:Val	Missense	Distal	Yes
55	Male	49	CCA:TCA	Pro:Ser	Missense	Distal	Yes
59	Male	67	CTG:CCC	Leu:Pro	Missense	Distal	Yes
59	Male	67	GTT:ATT	Val:Ile	Missense	Distal	Yes
59	Male	67	GTG:GGG	Val:Gly	Missense	Distal	Yes
54	Male	70	ATA:TAA	Ile:Stop	Nonsense	Distal	Yes
61	Male	91	AAA:delet	Lys:	Frameshift	Distal	No
61	Male	91	GGA:TGA	-	Intronic	Distal	No

Two mutations listed at the end of the table have not been reported previously.

intramucosal tumors, most of them (86%) had invasive adenocarcinoma. There was no mutation detected in control normal tissues taken from patients.

The frequency of mutation was increased in higher stages of tumor and this was statistically significant throughout all stages of cancer (p-value for trend=0.009). There was no significant association between the presence of mutation and either tumor location or histological differentiation of tumor (Table 1). The correlation between individual mutations and disease stages and also tumor grade were described in Table 3. Due to small number of each mutation, we were unable to apply any statistical test to confirm or reject associations.

The location of tumor in groups with and without mutated p53 was assessed. While the proportion of proximal tumor in mutated group appears to be lower than control group (21.4% vs 36.2%) but the difference was not statistically significant (Table 1). Details of point mutations by tumor location have been shown in Table 2.

1. IHC

Protein stability detected by IHC was found in the specimens of 11 (78.6%) patients with mutations. There were six (42.9%) samples with the highest proportion of cancerous cells which was indicated by maximum colour absorption. The highest proportion was designated as (3+) which shows that over 50% of cells being stained. Three (21.4%) samples were designated as (+2) indicating 25% to 50% stained cells and two (14.3%) samples were scored (+1) with 10% to 25% stained cell. Control samples, taken from nontumoral tissues of patient's colon, were scored as negative when the proportion of stained cells was less than 10% (Fig. 1). Three cases (21.4%) of cancer patients with mutation in exons 5 and 6 had no protein stability as IHC showed. There was no protein stability in specimens of cancer patients without p53 mutations. Therefore, presence of protein stability (all scores except 0) was significantly associated with presence of p53 mutation (p<0.001, Fisher's exact test).

Table 3. Association among p53 Mutations and the Stage of Disease and Tumor Grades in Patients with Colorectal Cancer

Mutation	Amino acid change	Disease stage	Tumor grade
GAT:TAA	Asp:Stop	C	Well differentiated
GGA:AGG	Gly:Arg	B	Well differentiated
AAA:AAT	Lys:Asn	B	Well differentiated
ATA:AAT	Ile:Asn	B	Well differentiated
GCT:GTC	Ala:Va	B	Well differentiated
TCA:TTC	Ser:Phe	A	Moderately differentiated
GCA:GGA	Ala:Gly	B	Moderately differentiated
TTT:TTA	Phe:Leu	B	Poorly differentiated
CCC:TCC	Pro:Ser	B	Well differentiated
GGG:GCG	Gly:Ala	C	Well differentiated
CCG:CTC	Pro:Leu	C	Well differentiated
CAG:CAC	Glu:His	A	Well differentiated
ACC:ATC	Thr:Ile	B	Well differentiated
GAT:GTG	Asp:Val	D	Well differentiated
CCA:TCA	Pro:Ser	B	Poorly differentiated
CTG:CCC	Leu:Pro	C	Moderately differentiated
GTT:ATT	Val:Ile	C	Well differentiated
GTG:GGG	Val:Gly	C	Well differentiated
ATA:TAA	Ile:Stop	B	Well differentiated
AAA:delet	Lys:	C	Well differentiated
GGA:TGA	-	C	Well differentiated

2. Survival determination

Patients' follow-up was started 6 months after diagnosis and continued up to 4 years. At the end of this period, there were twelve deaths among all patients: seven with mutations and five without any mutation. The presence of p53 mutation was significantly associated with lower survival rate than that those without mutations (log rank test, p<0.001) (Fig. 2). Similarly, patients with advanced stages of cancer (C and D) had significantly worse prognosis than that those in stages A and B (log rank test, p<0.001). To explore the effect of stage of cancer on survival function of p53 mutation, the analysis was reconducted after adjustment for cancer stage. Although subgroup with higher stage of cancer had shorter survival than those in lower stage but this finding did not change the survival difference of mutated p53 versus nonmutated groups (log rank test, p=0.003). There were no significant associations between survival of patients and either histological differentiation or location of tumor.

DISCUSSION

This is the first report of the identification of missense and

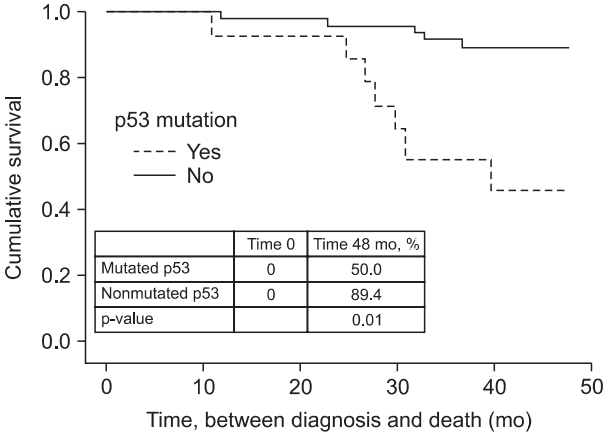

Fig. 2. Kaplan-Meier plot of survival in colorectal cancer patients with and without p53 mutations. The test was performed to determine the time between the first diagnosis of colorectal cancer and the time of death (due to colorectal cancer) during follow-up.

nonsense mutations in exons 5 and 6 of p53 gene in patients with colorectal cancer from Isfahan, Central Iran. The high frequency of p53 mutations in exons 5 and 6 in this population is similar to those from other ethnic origins. Although most of the 21-point mutations in our study were reported previously by other authors, but this study added two novel point mutations to medical literature for the first time. In addition, direct sequencing showed that in the three patients with primary colorectal cancer there were more than one mutation. We also showed very low rate of nonsense mutations.

The results of our study are consistent with several published studies from different populations. Yamashita *et al*.[16] showed p53 gene mutations in five out of 20 specimens (25%) from patients with sporadic colorectal cancer in Japan. Mahdavinia *et al*.[22] in a study conducted on a population from Northern Iran showed a total of 40% mutation rate of p53 gene, exons 5, or 6. The abnormal migration in exons 5 and 6 in the p53 gene has been described by Leahy *et al*.[29] in the 20% of 66 samples; of which there were seven abnormal bands in exon 5 and six were in exon 6. Contrary to our results, Pan *et al*.[24] reported only one mutation located in exons 5 and 6 in a group of 97 patients with rectal carcinoma.

Protein stability was another finding which was evident in 79% of our mutated specimens. The presence of protein stability in sample cancer cells has been regarded as a sign of mutation and IHC can be used for detection of p53 protein.[24] However, there were some limitations in utilization of IHC because it is useful only when a mutation can normally leads to protein stability. It means that IHC would not be useful to detect any nonsense silent mutation[33,34] due to absence or incomplete production of a protein. Substitution of stop codon with the normal codon or presence of a deletion in any exons which lead to protein production are possible explanations for this phenomenon. Therefore, relying only on IHC for diagnostic purposes may lead to an inappropriate treatment protocols in patients with colorectal cancer. This phenomenon may also happen in the opposite direction, in which occurrence of some protein stabilities may not due to mutation. We recommend all these possibilities to be considered in cancer laboratories and to be equipped with additional accurate molecular and sequencing techniques along with IHC.

Our findings showed that there was more than one mutation in three (5%) patients with colorectal cancer which is comparable to another study from Iran with same rate[22] and lower than the rate of 17% which has been reported by Leahy *et al*.[29]

We tried to investigate association between location of tumor and frequency of p53 mutations. Although there was a weak tendency toward higher frequency of mutations in distal tumors but the difference was statistically nonsignificant. A number of studies showed that the rate of mutations is higher in tumors of distal part compared to proximal colon.[8,35] This may be due to higher exposure of mucosa to more concentrated toxic substances contributing to cancer development in distal versus proximal colon.

Although the study did not focus on the role of gender, results suggested that the mutation rates in female were less than that in males (13% vs 26% in females and males, respectively), but this difference did not reach statistically significant level due to small number of female patients. Although underlying mechanism of the male predominance in the rate of p53 mutations is not clear, perhaps there are common explanations for male predominance in the carcinogenesis of colorectal cancer and parallel genetic alterations including p53 mutations.[32] Either excess exposure to some environmental risk factor among men or some protective endogen pathways among women are main factors to be investigated in future. Much stronger male predominance has been described in upper gastrointestinal tract adenocarcinoma and some protective endogen factors has been suggested previously.[36]

We were able to investigate the prognosis of cancer in patients with mutated versus nonmutated p53 gene. The mutated group had apparently shorter survival than group with wild type p53 gene in their tumor. While this finding is consistent with the results of several studies,[37-39] but association between p53 overexpression and prognosis has not been supported by a number of studies.[40,41] Considering the conflicting results on usefulness of p53 gene mutation for prediction of prognosis, and presence of robust clinicopathologic staging systems, i.e., Duke classification, a recent guideline did not support routine application of p53 gene mutation and some other tumor makers for prognostic purpose in colorectal cancer.[42]

In summary, although the current study suffers from relatively small number of cancer patients, which is reflected by some weak associations, it is first attempt to describe p53 gene mutation in colorectal cancer patients from Isfahan, Central Iran. Results of this study may help scientists to understand similarities and differences of p53 mutations among different populations with various life style behaviours and genetic backgrounds. It may also help clinicians to be conscious about magnitude of genetic alterations which are known to be of predictive value in clinical trials.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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