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

Rahim Golmohammadi*, Mohammad J. Namazi*, Mehdi Nikbakht†, Mohammad Salehi‡, and Mohammad H. Derakhshan†

†Faculty of Medicine, Sahevar University of Medical Sciences, Sahevar, ‡Faculty of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran, and †Section of Gastroenterology, Institute of Cardiovascular and Medical Sciences, University of Glasgow, Glasgow, UK

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.5,6 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.7,8 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.9,10 Mutations in the p53 gene prevented cell death in the DNA damaged cells which would be a reason for the resistance to chemotherapy.11,12 The p53 gene also acts as a transcription factor to regulate expression of more than 100 different genes.13

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 mutations in colorectal adenocarcinoma are detected in these 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...
70% respectively. 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, 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. 20

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% H2O2 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 x400 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). 21

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. 20 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’TGTTCACCTTGCCCTGTACT 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. 21 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
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%)
females. The higher number of male patients in our study may reflects male predominance of colorectal cancer in the population, 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...
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).

### 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...
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. showed p53 gene mutations in five out of 20 specimens (25%) from patients with sporadic colorectal cancer in Japan. Mahdavinia et al. 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. 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. 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. 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 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 patients with colorectal cancer which is comparable to another study from Iran with same rate and lower than the rate of 17% which has been reported by Leahy et al. We tried to investigate association between location of tumor and frequency of p53 mutations. Although there was 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. 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. Either excess exposure to some environmental risk factor among men or some protective endogenous 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 endogenous factors has been suggested previously.

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, but association between p53 overexpression and prognosis has not been supported by a number of studies. 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.

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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REFERENCES

1. Karsa LV, Lignini TA, Patrick J, Lambert R, Sauvaget C. The dimensions of the CRC problem. Best Pract Res Clin Gastroenterol 2010;24:381-396.
2. Jemal A, Center MM, DeSantis C, Ward EM. Global patterns of cancer incidence and mortality rates and trends. Cancer Epidemiol Biomarkers Prev 2010;19:1893-1907.
3. Yee YK, Tan VP, Chan P, Hung IF, Pang R, Wong BC. Epidemiology of colorectal cancer in Asia. J Gastroenterol Hepatol 2009;24:1810-1816.
4. Sadjadi A, Malekzadeh R, Derakhshan MH, et al. Cancer occurrence in Ardabil: results of a population-based cancer registry from Iran. Int J Cancer 2003;107:113-118.
5. Markowitz SD, Bertagnolli MM. Molecular origins of cancer: molecular basis of colorectal cancer. N Engl J Med 2009;361:2449-2460.
6. Cunningham D, Atkin W, Lenz HJ, et al. Colorectal cancer. Lancet 2010;375:1030-1047.
7. Gryfe R. Overview of colorectal cancer genetics. Surg Oncol Clin N Am 2009;18:573-583.
8. Russo A, Bazan V, Iacobetta B, et al. The TP53 colorectal cancer international collaborative study on the prognostic and predictive significance of p53 mutation: influence of tumor site, type of mutation, and adjuvant treatment. J Clin Oncol 2005;23:7518-7528.
9. Carson DA, Lois A. Cancer progression and p53. Lancet 1995;346:1009-1011.
10. Rogel A, Popliler M, Webb CG, Oren M. p53 cellular tumor antigen: analysis of mRNA levels in normal adult tissues, embryos, and tumors. Mol Cell Biol 1985;5:2851-2855.
11. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature 2000;408:307-310.
12. Ryan KM, Phillips AC,Vousden KH. Regulation and function of the p53 tumor suppressor protein. Curr Opin Cell Biol 2001;13:332-337.
13. Schmitt CA, Lowe SW. Apoptosis and chemoresistance in transgenic cancer models. J Mol Med (Berl) 2002;80:137-146.
14. Levine AJ, Hu W, Feng Z. The p53 pathway: what questions remain to be explored? Cell Death Differ 2006;13:1027-1036.
15. Zhao R, Gish K, Murphy M, et al. Analysis of p53-regulated gene expression patterns using oligonucleotide arrays. Genes Dev 2000;14:981-993.
16. Yamashita K, Yoshida T, Shinoda H, Okayasu I. Novel method for simultaneous analysis of p53 and K-ras mutations and p53 protein expression in single histologic sections. Arch Pathol Lab Med 2001;125:347-352.
17. Diez M, Pollan M, Miguérza JM, et al. Time-dependency of the prognostic effect of carcinoembryonic antigen and p53 protein in colorectal adenocarcinoma. Cancer 2000;88:35-41.
18. Chang SC, Lin JK, Lin TC, Liang WY. Genetic alteration of p53, but not overexpression of intratumoral p53 protein, or serum p53 antibody is a prognostic factor in sporadic colorectal adenocarcinoma. Int J Oncol 2005;26:65-75.
19. Lam AK, Ong K, Ho YH. hTERT expression in colorectal adenocarcinoma: correlations with p21, p53 expressions and clinicopathological features. Int J Colorectal Dis 2008;23:587-594.
20. Nunes BL, Jucá MJ, Gomes EG, et al. Metalloproteinase-1, metalloproteinase-7, and p53 immunoreexpression and their correlation with clinicopathological prognostic factors in colorectal adenocarcinoma. Int J Biol Markers 2009;24:156-164.
21. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. Science 1991;253:49-53.
22. Mahdavinia M, Bishehsari F, Verginelli F, et al. P53 mutations in colorectal cancer from northern Iran: relationships with site of tumor origin, microsatellite instability and K-ras mutations. J Cell Physiol 2008;216:543-550.
23. Nasierowska-Guttmejer A, Trzeciak L, Nowacki MP, Ostrowski J. p53 protein accumulation and p53 gene mutation in colorectal cancer. Pathol Oncol Res 2000;6:275-279.
24. Pan ZZ, Wan DS, Chen G, Li LR, Lu ZH, Huang BJ. Co-mutation of p53, K-ras genes and accumulation of p53 protein and its correlation to clinicopathological features in rectal cancer. World J Gastroenterol 2004;10:3688-3690.
25. Tang R, Wang PF, Wang HC, Wang JY, Hsieh LL. Mutations of p53 gene in human colorectal cancer: distinct frameshifts among populations. Int J Cancer 2001;91:863-868.
26. Munro AJ, Lain S, Lane DP. P53 abnormalities and outcomes in colorectal cancer: a systematic review. Br J Cancer 2005;92:434-444.
27. Tominaga T, Iwashashi M, Takifuji K, et al. Combination of p53 codon 72 polymorphism and inactive p53 mutation predicts chemoresensitivity to 5-fluorouracil in colorectal cancer. Int J Cancer 2010;126:1691-1701.
28. Zhang SQ, Wu JM, Su YY. Hangzhou colorectal cancer staging (modified Duke's classification). Chin Med J (Engl) 1983;96:675-680.
29. Leahy DT, Salmon R, Mulcahy H, Sheahan K, O'Donoghue DP, Parfrey NA. Prognostic significance of p53 abnormalities in colorectal carcinoma detected by PCR-SSCP and immunohistochemical analysis. J Pathol 1996;180:364-370.
30. Coombs NJ, Gough AC, Primrose JN. Optimisation of DNA and RNA extraction from archival formalin-fixed tissue. Nucleic Acids Res 1999;27:2x12.
31. Erster S, Slade N, Molloy LM. Mutational analysis of p53 in human tumors: direct DNA sequencing and SSCP. Methods Mol Biol 2003;234:219-230.
32. Mousavi SM, Gouya MM, Ramazani R, Davanlou M, Hajisadeghi N, Seddighi Z. Cancer incidence and mortality in Iran. Ann Oncol 2009;20:556-563.
33. Bazan V, Migliavacca M, Tubiolo C, et al. Have p53 gene mutations and protein expression a different biological significance in colorectal cancer? J Cell Physiol 2002;191:237-246.
34. Kato S, Han SY, Liu W, et al. Understanding the function-structure and function-mutation relationships of p53 tumor suppressor
protein by high-resolution missense mutation analysis. Proc Natl Acad Sci U S A 2003;100:8424-8429.
35. Slattery ML, Curtin K, Wolff RK, et al. A comparison of colon and rectal somatic DNA alterations. Dis Colon Rectum 2009;52:1304-1311.
36. Derakhshan MH, Liptrot S, Paul J, Brown IL, Morrison D, McColl KE. Oesophageal and gastric intestinal-type adenocarcinomas show the same male predominance due to a 17 year delayed development in females. Gut 2009;58:16-23.
37. Lim SC, Lee TB, Choi CH, Ryu SY, Min YD, Kim KJ. Prognostic significance of cyclooxygenase-2 expression and nuclear p53 accumulation in patients with colorectal cancer. J Surg Oncol 2008;97:51-56.
38. Molleví DG, Serrano T, Ginestà MM, et al. Mutations in TP53 are a prognostic factor in colorectal hepatic metastases undergoing surgical resection. Carcinogenesis 2007;28:1241-1246.
39. Chang SC, Lin JK, Yang SH, Wang HS, Li AF, Chi CW. Relationship between genetic alterations and prognosis in sporadic colorectal cancer. Int J Cancer 2006;118:1721-1727.
40. Theodoropoulos GE, Karafoka E, Papailiou JG, et al. P53 and EGFR expression in colorectal cancer: a reappraisal of 'old' tissue markers in patients with long follow-up. Anticancer Res 2009;29:785-791.
41. Anwar S, Frayling IM, Scott NA, Carlson GL. Systematic review of genetic influences on the prognosis of colorectal cancer. Br J Surg 2004;91:1275-1291.
42. Duffy MJ, van Dalen A, Haglund C, et al. Tumour markers in colorectal cancer: European Group on Tumour Markers (EGTM) guidelines for clinical use. Eur J Cancer 2007;43:1348-1360.