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Original scientific paper

THE LACK OF CORRELATION BETWEEN *TP53* MUTATIONS AND GASTRIC CANCER: A REPORT FROM A PROVINCE OF IRAN

Javad SAFFARI-CHALESHTORI[1], Mohammad-Amin TABATABAIEFAR[2], Payam GHASEMI-DEHKORDI[3], Effat FAROKHI[3], Mohammad-Taghi MORADI[3], Morteza HASHEMZADEH-CHALESHTORI[3]

[1] Clinical Biochemistry Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran
[2] Department of Medical Genetics, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran
[3] Cellular and Molecular Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran

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Gastric cancer ranks second cause of cancer death worldwide after lung cancer. Its etiology is heterogeneous and genetic factors including protooncogenes and tumor suppressor genes always contribute to the progression of cancer. The *TP53* tumor suppressor gene has a broad role in genomic stability and DNA repair. The aim of this study was to determine the *TP53* gene mutations in gastric cancer specimens in Chaharmahal Va Bakhtiari province of Iran. In this descriptive-lab based study, we investigated the promoter and exons of *TP53* gene mutations in 38 paraffin-embedded gastric cancer specimens. DNA was extracted following a standard phenol-chloroform protocol. The *TP53* gene mutations were determined using PCR-SSCP & PCR-RFLP procedures. The present study revealed no *TP53* gene mutation in the promoter and exons in the gastric cancer subjects studied. While *TP53* gene mutations have been reported as the most frequent genetic alterations and are found in about 50% of the human malignancies, no mutation was detected in this study. This may be due to mutations in other related genes in the same pathway or epigenetic factors.

Key words: gastric cancer, Iran, PCR-SSCP, RFLP, *TP53* gene

Corresponding author: Morteza Hashemzadeh-Chaleshtori, Cellular and Molecular Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Rahmatieh, Shahrekord, Iran, Postal code: 8813833435, Tel: +98-38-33346692. Fax: +98-38-33330709. E-mail: molecularskums@gmail.com

INTRODUCTION

Gastric cancer is the main cause of cancer death worldwide (HOWSON *et al.*, 1986). Gastric cancer comprises the second most frequent cancer worldwide and the fourth in Europe (GONZALEZ *et al.*, 2002). The two type of gastric cancer include the intestinal and peripheral, or the so-called intestinal, types (LAUREN, 1965; KATIYAR *et al.*, 2000). Although environmental factors such as *Helicobacter pylori* (*H. pylori*) infections are considered to be of importance for the familial clustering of gastric carcinoma (HEMMINKI and JIANG, 2002), gastric cancer is complex in nature and could involve many genes and their interaction with environmental factors. Proto-oncogenes and Tumor suppressor genes such as *TP53* are important in carcinogenesis (CHO *et al.*, 2005).

The transcription factor p53 responds to diverse cellular stresses to regulate target genes that induce cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism. P53 appears to induce apoptosis through non-transcriptional cytoplasmic processes (TOLEDO *et al.*, 2006). P53 has an important role in transcriptional regulation and cytoplasmic functions (GREEN and KROEMER, 2009). This gene is involved in cell cycle control (QIAN *et al.*, 2008) and acts to decrease the angiogenesis (SANO *et al.*, 2007). Alteration or inactivation of *TP53*, the gene encoding p53, by mutation or by interaction with oncogene products of DNA tumor viruses can lead to cancer (LEVINE *et al.*, 1991). Post translation processes such as acetylation (TANG *et al.*, 2008), phosphorylation (TAIRA *et al.*, 2007) and methylation (SHI *et al.*, 2007) regulate p53 function. P53 limits malignant progression by suppressing the acquisition of further alterations that lead to tumor progression (FELDSER *et al.*, 2010).*TP53* is on the short arm of chromosome 17 (BENCHIMOL *et al.*, 1985). It contains 11 exons, 2 transcriptional start sites in exon 1, and alternative splicing sites in intron 2 and between exons 9 and 10 and transcription initiation site in intron 4 (BOURDON *et al.*, 2005). There are two promoters in the *TP53* gene. The first one is located 100 to 250 bp upstream of the non-coding first exon, and the second, a stronger promoter, is located within the first intron (REISMAN *et al.*, 1988). Exon 1 and a part of the end exon 11 are non-coding but other regions are codings (TOMMASI *et al.*, 2001). The hotspot regions of *TP53* are in 5-8 exons and more than 90% of mutations are in three exons. This region encompasses codons 110 to 307 (KATIYAR *et al.*, 2000; TOMMASI *et al.*, 2001).

So far, there have been many studies confirming a role for p53 in the carcinogenesis of gastric cancer. There are, however, other studies which fail to confirm such relation.

In the present study, PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) technique was applied to detect two “hot spot” sites within exons 6 and 8 previously associated with gastric cancer. We also used polymerase chain reaction (PCR)-single stranded conformation polymorphism (SSCP) to rapid and sensitive detection of other mutations of *TP53* gene in the promoter and exons 2 – 11 of the *TP53* gene.

MATERIALS AND METHODS

Sampling and DNA extraction

The study was approved by the Review Board of Shahrekord University of Medical Sciences. Thirty-eight samples of paraffin-embedded gastric cancer archive tissues held in the central lab of Shahrekord University affiliated hospital (Kashani hospital) that collected from Chaharmahal Va Bakhtiari province of Iran, were recruited in this study (Figure 1). The average age of patients was about 67 years old. Thirty patients were male (51-77 years of age) and the rest were female (38-70 years of age). Totally, nine samples were diffuse and 29 were intestinal types

as well as 24, 8 and 6 of patients were in primary, advanced, and localization status of cancer. The patients' groups were homogenous and all samples were collected at the time of diagnosis.

Figure 1. The geographical position of Chaharmahal Va Bakhtiari province (southwestern Iran) that this study performed.

For DNA extraction, 25 mg of samples were deparaffined with Xylol & Ethanol and a standard phenol-chloroforms method was used. DNA concentration and purity was measured by spectroscopy (UNICO 2100, USA) (GRIMBERG *et al.*, 1989).

PCR amplification

PCR primers were designed using a primer 3 website (http://frodo.wi.mit.edu/) (Table 1). The promoter and exons 2 to 11 of the *TP53* gene were amplified by PCR.

The PCR amplification was performed in a total volume of 25 µL mixture containing: 100 ng genomic DNA, 1.0 µM of each primer, 200 µM of each dNTP, 2.0 µM of $MgCl_2$ and 1.0 U *Taq* DNA polymerase and 10 µL *Taq* buffer (Fermentas, Germany) using the Astec gradient 96 Thermocycler (Astec, Japan).

The thermal cycle profile was as follows: initial denaturation at 95°C for 3 min, followed by 36 cycles including: 95°C for 1 min, annealing temperature for 30 sec for different primers (55°C - 63°C) and extension at 72°C for 30 sec (Table 2). A final extension step was followed at 72°C for 8 min. All of the PCR products were analyzed on 8% poly acrylamide gel electrophoresis (PAGE). PCR products were visualized by means of silver (AgNo3) staining.

Table 1. PCR primers of p53 *designed in this study*

Primers name	Primers Sequence	Tm	Product length (bp)
P_{pro}-F	5′-TCTGGGAGAAAACGTTAGGG-3′	57.3	
P_{pro}-R	5′-CTGGCACAAAGCTGGACAGT-3′	59.4	345
P_{pro}m	5′-TCTGGGAGAAAACGTTT*GGG-3′	57.3	
P2-F	5′-ATCCCCACTTTTCCTCTTGC-3′	57.3	
P2-R	5′-TCCCACAGGTCTCTGCTAGG-3′	61.4	198
P2m	5′-ATCCCCACTTTTCCTT*TTGC-3′	55.3	
P3-F	5′-CCATGGGACTGACTTTCTGC-3′	59.4	
P3-R	5′-GGGGACTGTAGATGGGTGAA-3′	59.4	170
P3m	5′-CCATGGGACTGACTTC*CTGC-3′	61.4	
P4-F	5′-CCTGGTCCTCTGACTGCTCT -3′	61.4	
P4-R	5′-GCCAGGCATTGAAGTCTCAT-3′	57.3	360
P4m	5′-CCTGGTCCTCTGAT*TGCTCT-3′	59.4	
P5-F	5′-GACTTTCAACTCTGTCTCCT-3′	55.3	
P5-R	5′-CTGGGGACCCTGGGCAACCA-3′	65.5	270
P5m	5′-GACTTTCAACTCTGTT*TCCT-3′	53.2	
P6-F	5′-GTCCCCAGGCCTCTGATTC-3′	61	
P6-R	5′-CTTAACCCCTCCTCCCAGAG-3′	61.4	190
P6m	5′-GTCCCCAGGCCTCTA*ATTC-3′	58.8	
P7-F	5′-TTATCTCCTAGGTTGGCTCT-3′	55.3	
P7-R	5′-CAAGTGGCTCCTGACCTGGA-3′	61.4	136
P7m	5′-TTATCTCCTAGGTTA*GCTCT -3′	53.3	
P8-F	5′-CCTTACTGCCTCTTGCTTC-3′	56.7	
P8-R	5′-TGAATCTGAGGCATAACTGC-3′	55.3	240
P8m	5′-CCTTACTGCCTCTC*GCTTC-3′	58.8	
P9-F	5′-TTGCCTCTTTCCTAGCACTG-3′	57.3	
P9-R	5′-CCCAAGACTTAGTACCTGAA-3′	55.3	100
P9m	5′-TTGCCTCTTTCCTAGT*ACTG-3′	55.3	
P10-F	5′-CTCTGTTGCTGCAGATC-3′	52.8	
P10-R	5′-GCTGAGGTCACTCACCT-3′	55.2	140
P10m	5′-CTCTGTTGCTGCG*GATC-3′	55.2	
P11-F	5′-TGTCATCTCTCCTCCCTGCT-3′	59.4	
P11-R	5′-CAAGGGTTCAAAGACCCAAA-3′	55.3	205
P11m	5′-TGTCATCTCTCCTCCT*TGCT-3′	57.3	

(P: Primer, Pro: Promoter, F: Forward, R: Reverse, Pm: mutant Primer (Forward), Tm: Temperature Melting for primers)

*nucleotide replaced in forward primer

Table 2. Thermal cycle profile of the TP53 gene exons

Exons	Denaturation	Time	Annealing	Time	Extension	Time
Exon 2	96ºC	1 min	61.5ºC	1 min	72ºC	1 min
	96ºC	40 sec	58ºC	30 sec	72ºC	40 sec
Exon 3	96ºC	30 sec	63ºC	30 sec	72ºC	30 sec
Exon 4	96ºC	1 min	57ºC	40 sec	72ºC	50 sec
Exon 5	96ºC	1 min	62.5ºC	1 min	72ºC	1 min
	96ºC	40 sec	59ºC	30 sec	72ºC	40 sec
Exon 6	96ºC	30 sec	61ºC	30 sec	72ºC	30 sec
Exon 7	96ºC	30 sec	57ºC	30 sec	72ºC	30 sec
Exon 8	96ºC	45 sec	54ºC	40 sec	72ºC	50 sec
Exon 9	96ºC	30 sec	58ºC	30 sec	72ºC	30 sec
Exon 10	96ºC	30 sec	55ºC	30 sec	72ºC	30 sec
Exon 11	96ºC	30 sec	62ºC	30 sec	72ºC	30 sec
Promoter	96ºC	50 sec	59ºC	40 sec	72ºC	40 sec

SSCP and HA and DNA sequencing

In this study, due to financial constraints first for determination of polymorphisms single-stranded conformation polymorphism (SSCP) were done on the samples and for detection of the difference between polymorphisms and mutations, RFLP using specific restriction enzymes were performed. For SSCP, a mixture of 5 µL of PCR product and 4 µL of denaturing buffer (90% formamide, 10 mM disodium EDTA, 1% xylene cyanol, and 1% bromophenol blue) was heated at 95˚C for 15 minutes and then immediately placed on ice to prevent renaturation (KROTHAPALLI *et al.*, 2012).

We used site-directed mutagenesis using primers, for the promoter and exons, changed in the 3´end of forward primers for one nucleotide (C to T or T to C) used in the healthy samples to create positive controls for SSCP.

For heteroduplex analysis (HA), 2.2 µL of PCR product from each sample was mixed with 3.2 µL EDTA (5.0M), and heated at 95˚C for 5 minutes and was slowly cooled using 60 cycles of 30 sec to 37˚C. Samples (5 µL) of each denatured PCR product were loaded on non-denaturing PAGE (8%) for 1 h at 50mA (Merk, Germany) (Table 3). The prepared SSCP product was mixed with HA product for each sample and was loaded on 8% PAGE. Bands were visualized by means of silver staining. Samples with mobility shifts were verified by a second independent PCR-SSCP.

DNA sequencing of the PCR-amplified product with the motility shift on the gel and hose made positive controls was carried out bi-directionally on an ABI 3130 automated sequencer (Applied Biosystems) using the same primers.

Table 3. The optimized conditions for SSCP electrophoresis

Exons	Gel concentration	Time (h)	Milliampere (MA)	Voltage (V)	Temperature (ºC)	Other
Exon 2	8%	5.30	32	280	20	—
Exon 3	10%	6.30	32	280	20	—
Exon 4	6%	14	13	120	4	urea10g
Exon 5	6%	14	13	120	4	urea10g
Exon 6	12%	7	32	280	20	—
Exon 7	12%	6	32	280	20	—
Exon 8	8%	5.30	32	280	20	—
Exon 9	6%	3.50	32	280	20	urea10g
Exon 10	12%	6	32	280	20	—
Exon 11	12%	7	32	280	20	—
Promoter	12%	10	32	280	20	glycerol 80%

PCR-RFLP

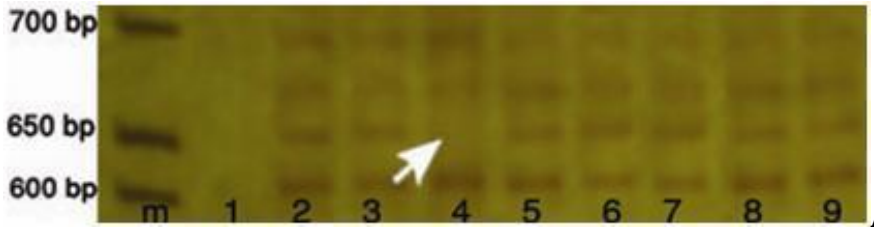
In this study, we used PCR-RFLP in codon 248 with CGG sequence in exon 7 and codon 282 in exon 8 with CGG sequence for which mutations have been frequently reported in gastric cancer.

The restriction enzyme *Hpa*II (contain: a mixture of 10 μL of PCR product, 1 μL restriction enzyme, 2 μL buffer and 7 μL dH_2O) digests (for 12 h at 37°C) within the sequence corresponding to the codon (CGG) at position 248 and codon (CGG) at position 282 to generate two visible fragments of 82 and 54 bp for codon 248 and fragments 140 and 100 bp for codon 282 and leaves the mutant codons uncut. After electrophoresis in 8% poly acryl amide gel electrophoresis (PAGE) in 120 V for 1 h, it was visualized by means of silver ($AgNO_3$) staining.

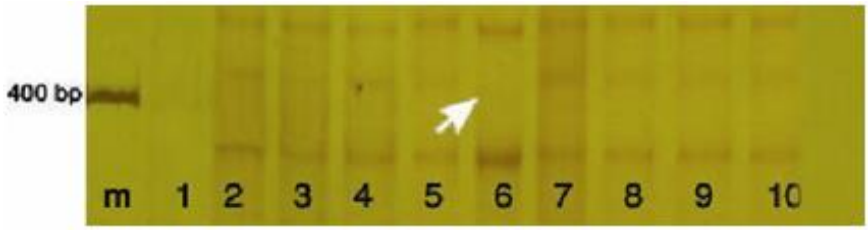
RESULTS

SSCP analysis showed the differential motility of electrophoretic bands for the positive controls in the denatured region. This was shown for the positive controls of all exons. For the patients' samples, no shifted band was evident except for three cases for exon 8 in which upon DNA sequencing no mutation was found (Figure 2).

PCR-RFLP results for codon 248 in exon 7 and codon 282 in exon 8 showed no mutation in the relative sequences, either. All the samples showed a single cut in exon 7 in 82 bp region and a single cut in exon 8 producing a 140 bp band (Figure 3). Thus, no mutation was found for any of the cancer samples (Figure 4).



A.



B.

Figure 2. The results of PCR-SSCP on promoter and exon 8 for example (m: Marker 100 bp and 1= negative control sample without denaturated PCR product)
A: Promoter: 4= positive control, 2-3,5-9=Patient samples without the mutation.
B: Exon 8: 6= positive control, 2-5,7-9=Patient samples without the mutation.

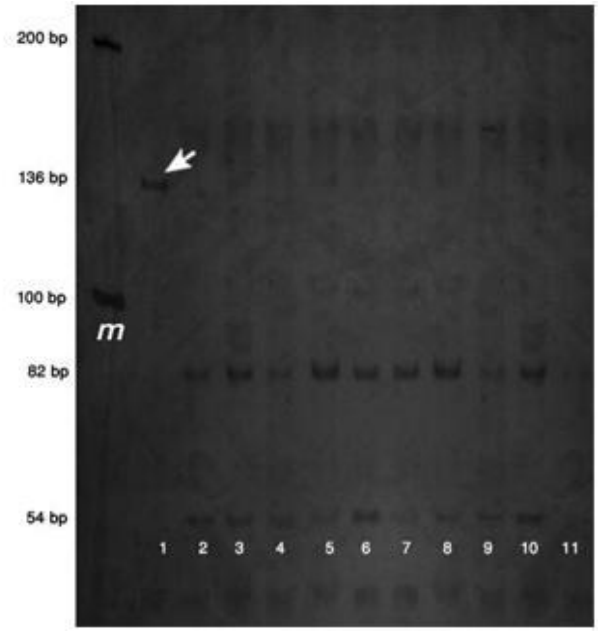


Figure 3. The results of PCR-RFLP (m: marker 100 bp, (1) PCR product without restriction Enzyme, (A): codon 248 in exon 7: All the samples digested.

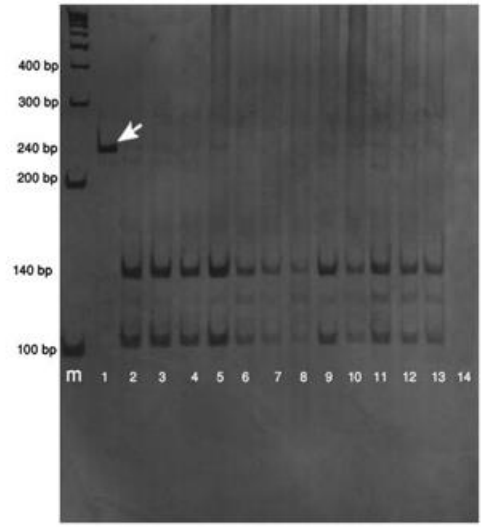


Figure 3. The results of PCR-RFLP (m: marker 100 bp, (1) PCR product without restriction Enzyme, (B): codon 284 in exon 8: All of samples digested.

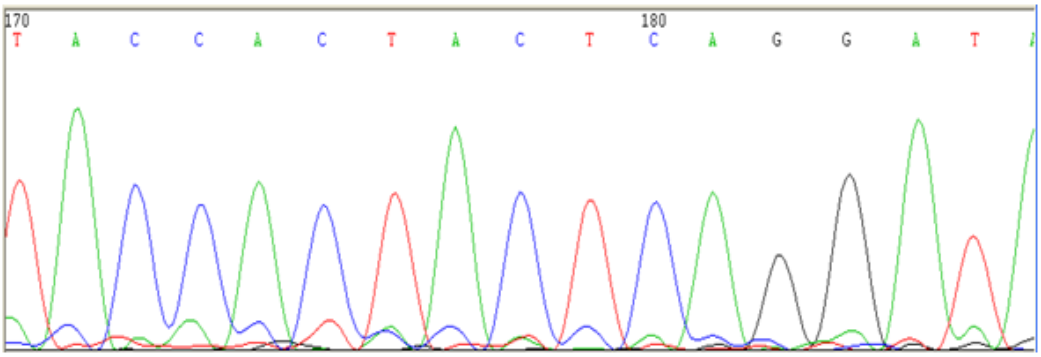


Figure 4. Sequencing of exon 8 of *TP53* in some of the samples with positive SSCP results which showed no mutation

DISCUSSION

The *TP53* tumor suppressor gene is among the most frequently mutated genes in human cancer (HIYAMA *et al.*, 1998). Many studies have presented clues of *TP53* alterations, including both mutations and polymorphisms, as the most widespread genetic changes in human cancers, such as gastric cancer, cervical cancer, breast cancer, lung and colorectal cancer (OKI *et al.*, 2005). Gastric adenocarcinomas have been shown to normally contain 18% to 58% *TP53* mutation (MAESAWA *et al.*, 1995; MONIG *et al.*, 1997). Although p53 nuclear staining can be seen in both intestinal and diffuse type gastric tumors, it is more commonly observed in the intestinal type (FENOGLIO-PREISER *et al.*, 2003). However, some investigators failed to find any difference between the two types (TANG *et al.*, 2010).

The existence and distribution of mutations in the gene differ among different cancers. For example, hepatocellular carcinoma is associated with codon 249 in exon 7 while no mutation in the codon has ever been reported for gastric cancer (MULLER and BORCHARD, 1996). While, researchers have shown an association of the *TP53* codon 72 polymorphism with colorectal cancer in South West Iran (DOOSTI *et al.*, 2011), a mutation in other codons might be more important in gastric carcinoma. The most common mutations reported thus far in *TP53* in relation to gastric cancer include codons 175 and 248 which we studied by the sensitive RFLP technique and found no mutation (ARREGUI *et al.*, 2002). The reported incidence of *TP53* mutations in invasive carcinomas ranges from 0-79% (YAMADA *et al.*, 1991). Meng and colleagues showed that mutation in exons 5-8 of the *TP53* gene is 61% in mammary carcinoma (MENG *et al.*, 1999). Codon 72 of the *TP53* gene mutations in breast cancer in the Iranian patients and reported that polymorphism in this codon to be associated with breast cancer in these patients (DOOSTI *et al.*, 2011). In their study, 70 out of 135 (51.85%) patients with breast cancer were heterozygous (Arg/Pro), 52 (38.52%) were homozygous for arginine (Arg/Arg) and 13 (9.63%) samples homozygous for proline (Pro/Pro) (TESTINO *et al.*, 2002). Studying 40 colorectal samples from Isfahan province, central Iran, 7 mutations in exon 6 of the gene were found (GOLMOHAMMADI and NIKBAKHT, 2006). The esophageal cancer study from north of Iran, a focal region of esophageal cancer, on 74 samples led to the finding of 54 (65%) positive samples for mutations in the gene (BIRAMIJAMAL *et al.*, 2001). Geographic region and ethnicity can affect the mutation distributions of genes. This has been shown for *TP53* mutations associated with gastric cancer. In Japan, for example, mutations of exon8 comprised 5.2% in a Japanese society while was obtained to be 28% in another population from Japan (MUNOZ-FONTELA *et al.*, 2005).

CHEN *et al.* (2011) studied exons 5-8 of *TP53* and reported 29.3% mutations in China. Shiao *et al*. (1994) studied in gastric cancer gene mutations and reported that the mutation in this gene was found in about 66% of the cases (SHIAO *et al.*, 1994). In contrast, LUINETTI *et al*. (1998) showed that mutation in this gene in gastric cancer is about 26%.

UCHINO *et al.* (1993) showed 25% mutations in primary gastric cancer and 42% mutation in advanced gastric cancer. TOLBERT *et al*. (1999) observed mutations in 42% of the intestinal and 21% of the peripheral gastric cancer. However, in 2005 40.6% mutation in intestinal and 8.2% in peripheral gastric cancer observed (OKI *et al.*, 2005). Based on a study by YAMADA *et al.* (1991) on 19 primary gastric cancers from Japan, no *TP53* mutation was reported. As mentioned above, there are many studies showing that mutations in the *TP53* are abundant in various cancers in the world. However, in our study analyzing 38 samples of paraffin-embedded tissue in gastric cancer in a province of Iran (Chaharmahal Va Bakhtiari) we failed to find any mutation in TP53 by PCR-SSCP techniques. Although we applied some modifications to the technique and used the simultaneous heteroduplex analysis, SSCP is not at all 100% sensitive and it is possible that some of the mutations might have been overlooked (WEGHORST *et al.*, 1993). We confirmed our study by PCR-RFLP techniques in codons 248 and 282 on exons 7 and 8 that are two hot spots regions for *TP53* in gastric cancer. Notably, all of the positive control samples in PCR-SSCP had shifted in gel electrophoresis. The restriction enzyme in PCR-RFLP cut all of the patient samples in codons 248 and 282 on exons 7 and 8.

In summary, in the present study the relation between gastric cancer and *TP53* gene, no mutation was detected. This could be tightly linked to the ethnicity. Other genes in the same pathway or alternatively, other pathways might be involved in the carcinogenesis process. Further studies with larger sample size from the region may better clarify the idea.

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NEDOSTATAK KORELACIJE IZMEĐU *TP53* MUTACIJA I KANCERA ŽELUCA: IZVEŠAJ IZ IRANSKE PROVINCIJE

Javad SAFFARI-CHALESHTORI[1], Mohammad-Amin TABATABAIEFAR[2], Payam GHASEMI-DEHKORDI[3], Effat FAROKHI[3], Mohammad-Taghi MORADI[3], Morteza HASHEMZADEH-HALESHTORI[3]

[1] Klinički istraživački centar za biohemiju, Basic Health Sciences Institute, Shahrekord Univerzitet Medicinskih nauka, Iran

[2] Departman Medicinske Genetike, Medicinska škola, Isfahan Univerzitet Medicinskih nauka, Isfahan, Iran

[3]Ćelijski i molekularni istraživački centar, Basic Health Sciences Institute, Shahrekord Univerzitet Medicinskih nauka, Iran

Izvod

Kancer želuca je na drugom mestu po smrtnosti u svetu, posle kancera pluća. Njegova etiologija je heterogena i genetski faktori uključujući protoonkogene i gene supresore tumora, uvek doprinosi razvoju kancera. Tumor supresor gen-*TP53* ima veliku ulogu u genomskoj stabilnosti i reparaciji DNK. Cilj ovog istraživanja bio je da se odrede mutacije gena *TP53* kod uzoraka kancera želuca u Iranskoj provinciji Chaharmahal Va Bakhtiari. U ovom laboratorijskom istraživanju, proučavani su promoter i egzoni *TP53* genske mutacije kod 38 uzoraka kancera želuca. Ekstrakcija DNK je urađena prema standardnom fenol-hloroform protokolu. Mutacije gena *TP53* određene su tehnikama PCR-SSCP i PCR-RFLP. Istraživanjem nisu otkrivene mutacije *TP53* gena u promotoru i egzonu uzoraka kancera želuca. Iako su mutacije gena *TP53* najčešće i utvrđene kod oko 50% maligniteta, u ovom istraživanju nisu utvrđene mutacije. Razlog mogu biti mutacije u drugim srodnim genima ili epigenetski faktor.

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