

Detection of p53 exon 9 gene mutation in bladder cancer by polymerase chain reaction-single-strand conformation polymorphism method

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ABSTRACT

Introduction: Urinary bladder cancer is the fourth most common diagnosed malignancy in men and the tenth in women worldwide. The successful treatment depends on early detection and specific diagnostic approaches. Mutations in the p53 tumor suppressor gene (TP53) are very common among the cancers, especially in bladder cancer that p53 mutation is identified in around 60 percents. **Aims:** In this study, we aim to detect p53 mutation in bladder cancer patients in Kermanshah city of Iran. **Materials and Methods:** Thirty paraffin-embedded specimens were obtained from bladder cancer patients. They were reviewed by pathologists for substantial amounts of neoplastic tissue. PCR-SSCP technique was used followed by standard silver staining to analyze TP53 exon 9 gene mutations in tumor samples. **RESULTS:** Polyacrylamide gel analysis represented mutations in 2 cases (6.7%) that were classified as p53-positive, and there were no mutations in 28 cases (93.3%) that were classified as p53-negative. No significant correlation observed between p53 mutation and histological grade, tumor stage, muscular propria status, patient sex, and age. **Conclusion:** These data are the first report on the p53 abnormalities in bladder cancer patients from Kermanshah. We can suppose that the percentage of p53 exon 9 mutation is low in bladder cancer patients from western Iran. However, further studies on a large number of tissue specimens, if possible, help to verify the obtained results.

Key words: Bladder cancer, mutation, p53, polymerase chain reaction, single-strand conformation polymorphism

INTRODUCTION

Transitional cell carcinoma (TCC) of the urinary bladder cancer is the most common bladder cancer subtype, which represents nearly 90% of all bladder cancers and is the 4th widespread cancer in men and the 9th among women around the world, and occurrence in men is three times more than in women. It is estimated that direct relation exists between bladder cancer occurrence and age in such a way that the common time of diagnosis is around 70 for both sexes.^[1,2]

Successful treatment of bladder cancer carcinoma depends on specific diagnostic approaches and early detection. Therefore, improving molecular techniques such as polymerase chain reaction (PCR) and single-strand conformation polymorphism (SSCP) would undoubtedly develop understanding of molecular abnormalities related to urothelial malignant transformation.^[3-8]

For instance, evaluating the archived paraffin-embedded tissue of patients who had bladder cancer can be helpful to recognize the prognostic significance of molecular events in bladder cancer.

P53 as a well characterized tumor repressor gene is mutated in almost 50% of most human cancers.^[7-9] Based on several studies regarding urothelial tumor, p53 mutation and p53 pathway inactivation are the most frequent documented genetic alterations in superficial and invasive bladder cancer. Besides, it has been reported that p53 is generally mutated in 60 percents of bladder cancers.^[9-11]

P53 is encoded by TP53 gene, which is located on the 17p13.1 chromosome and consists of 11 exons of about 20 kb.^[12,13]

Some of the mutations in p53, which are associated to cancer, mostly occur in DNA-binding domain and may prevent or abolish p53 basic role as guardian of the genome. This will affect p53 role for promoting apoptosis, mediating cell cycle arrest, DNA repair, and other responses to cell stress and DNA damage. So, this malfunction leads to cancer growth and progression.^[12-15]

Being relatively an early event in bladder tumors, p53 mutation cannot be detected in the morphologically normal urothelium.^[16]

The pattern of p53 mutation has been studied in bladder cancer: Some of p53 mutations are related to specific carcinogens, others may occur spontaneously, particularly G:C to A:T transitions, which are reported as a prevalence mutation in this cancer.^[17,18] Although several researches have shown that there is an approximately strong association between bladder cancer and p53 mutation (p53 mutation in 60% of bladder cancer), there is not enough information about the frequency of mutation in all exons of p53 tumor suppressor gene in this cancer. In this study, we choose p53 exon 9, on which less research has been performed, especially among Iranian people. The present study, which is the first report from Kermanshah, was planned to detect the rate of p53 mutation in bladder cancer in this city. Moreover, pathological parameters of bladder patients are assessed in correlation with p53 mutation.

MATERIALS AND METHODS

Samples

Patients with histologically confirmed TCC of the urinary bladder were enrolled from two Referral Urology Clinics at Kermanshah city. The number of patients was 30, and the mean age of diagnosis was 61 (range of 24-90 years). Tumor tissues were almost all newly diagnosed cases of urinary bladder cancer

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in Kermanshah city. They were almost the entire cases that occurred in the area recently. All etiological information available as pathology reports were requested from the treating physician/pathology laboratories. All cases were screened for mutation using PCR, nested PCR, and SSCP, in the region coding for exon 9 of the p53 gene.

Tissue

The tissue samples were obtained from formalin-fixed, paraffin-embedded specimens in the pathology department archives.

Control sample was DNA sample of normal people without a history of cancer. There were 30 cases, in which 24 (80%) were men and 6 (20%) were women in the specimens. Sections of 10 μm were cut from paraffin-embedded tumor blocks, stained with hematoxylin and eosin, and then reviewed by a pathologist to verify the tumor lesions and classify them according to the WHO and TNM guidelines. All tumors were staged and graded, and the information was available for each patient, which is shown in Table 1.

DNA extraction

Twenty sections of 10 μm thickness were sliced from each paraffin-embedded block and placed in 2 ml Eppendorf tube. Deparaffinization was performed twice with xylene, followed by one 95% and one 70% ethanol rinses. The samples were washed twice with PBS, and the pellets were re-suspended in 3-4 ml of digestion buffer containing 1 M Tris-HCl, 0.5 M EDTA, and 10% SDS. They were then incubated at 50°C in a water bath overnight. After the incubation, samples were re-suspended with 20 mg/ml proteinase K and incubated at 50°C once more overnight. Then, salting out of the cellular proteins was performed by dehydration and precipitation with a saturated 6 M NaCl solution. DNA was precipitated with cold Isopropanol and washed twice with cold 70% ethanol. The final solution was centrifuged, and the supernatant was dissolved in TE and then stored at 4°C.

PCR

Tumors were screened for mutations in exons 9, which includes the DNA-binding domain of the encoded protein. Primers for exon 9 are shown in Table 2. The length of PCR products of exon 9 was 174. PCR was carried out in a 50 μl reaction mixture, containing 1-3 μl of tumor lysate DNA (100 ng), 300 nM of each primer, 1 μl cetus buffer, 200 μM of each deoxyribonucleotide, 1.5 mM MgCl_2 , and 1.25-2.5 units of AmpliTaq DNA polymerase. The final volume was adjusted to 50 μl by using distilled water. All reactions contained a non-template control. The mixture

was overlaid with mineral oil and was amplified for 1 cycle at 94°C for 4 min, 55°C for 1 min, and 72°C for 1 min; followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and a final extension cycle of 94°C for 1 min and 60°C for 10 min. A preparation without DNA was included as a negative control. PCR products were resolved by electrophoresis in non-denaturing 2% agarose gel and visualized with ethidium bromide staining under UV light.

Nested PCR

An aliquot of each PCR products from the first PCR of exon 9 p53 were used as template and amplification was carried out in 50 μl reactions again, by using internal nested primers for exon 9 that are demonstrated in Table 2. The length of PCR products of exon 9 in nested PCR was 157 [Figure 1 and Table 3]. Cycle conditions were as described for the first PCR. Nested PCR products as well as the first PCR products were investigated by 2% agarose gel and envisaged with ethidium bromide staining under UV light.

SSCP analysis of the p53 gene

- Denaturation of PCR product
- Gel conditions

To improve the resolution of exon 9 bands, aliquots of 5-9 μL of the SSCP-PCR products were digested with 5 units of running solution (containing denaturing buffer, 95% formamide, 1% Boromo phenol blue, 5 M NaoH and 0.1% xylene cyanol) heated and denatured at 94°C for 5 min and immediately chilled on ice to prevent DNA renaturation, and hence approximately 10 μl was loaded per well onto 10% polyacrylamide non-denaturing gel (30:0.8) with 0.5 mm thickness. Electrophoresis was carried out at 4°C. Each gel included two kinds of negative controls including single-strand wild-type p53 and double-strand wild-type p53. For preparing double-strand, wild-type p53 denaturing solution without NaoH was employed. Besides, it was not heated as well. Electrophoresis was run with voltages of 140 for around 4 h. To increase the sensitivity of the screen and to prevent false-positive results, all samples were run three times under the same sets of gel conditions. Gels were subjected to silver staining after electrophoresis to visualize the bands of screened PCR products. Samples were compared with controls for the location of bands on the polyacrylamide gel. Therefore, mutations were detected as mobility shifts.

Statistical analysis

The relationship between p53 mutational status and the clinico-pathological variables was analyzed by the χ^2 -test or Fisher's exact test by using SPSS software.

RESULT

PCR-SSCP

Thirty paraffin-embedded bladder cancer samples from 30 patients were studied, in which 24 (80%) were men and 6 (20%) were women, and the mean age of diagnosis was 61 (range of 24-90 years).

Exons 9 of the p53 gene were screened for the presence of mutations by PCR-SSCP. Since the quantity of amplifiable DNA obtained from bladder tumor specimens were generally low, a sensitive nested PCR method was also developed for DNA amplification. The efficiency of the second round of amplification in nested PCR was enhanced because of the more rapid and more

Table 1: P53 mutation status

Pathology report	Number (%)
Histology	
Transitional cell carcinoma	30 (100)
Grade	
Low-grade (lowly malignant neoplasms)	22 (73.3)
High-grade (invasive highly malignant neoplasms)	8 (26.7)
Stage	
I	3 (10)
II	21 (70)
III	4 (13.3)
IV	2 (6.7)
Muscular propria involvement	
Involved	12 (40)
Not involved	18 (60)

Table 2: PCR primers

	Primer sequence	Region amplified	Product size	Annealing temperature
P53 Exon 9 sense	GCCTCAGATTCACCTTTTATCACC	26 bases upstream to exon 9	175	58.9
P53 Exon 9 antisense (internal)	CATTTTGAG TGTTAGACTGG	38 bases downstream to exon 9		57.6
P53 Exon 9 sense	ATCACCTTTCCTTGCCTC T	7 bases upstream to exon 9	158	54.5
P53 Exon 9 antisens	CATTTTGAGTGTTAGACTGG	38 bases downstream to exon 9		57.6

PCR=Polymerase chain reaction

Table 3: Grade results

	Result		Total
	Negative	Positive	Negative
Grade 1/4 count	3	0	3
% within grade	100.0	0.0	100.0
% within result	10.7	0.0	10.0
2/4 count	19	2	21
% within grade	90.5	9.5	100.0
% within result	67.9	100.0	70.0
3/4 count	4	0	4
% within grade	100.0	0.0	100.0
% within result	14.3	0.0	13.3
4/4 count	2	0	2
% within grade	100.0	0.0	100.0
% within result	7.1	0.0	6.7
Total count	28	2	30
% within grade	93.3	6.7	100.0
% within result	100.0	100.0	100.0

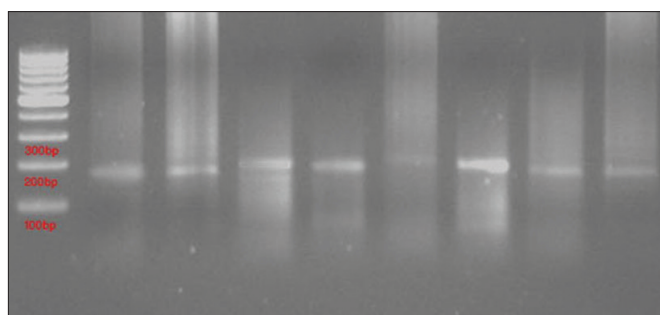


Figure 1: Nested PCR of DNA extracted from a paraffin wax-embedded section. The lanes show amplification of 157 base pair of p53 exon 9 gene

complete denaturation of the first reaction product compared to the total genome utilized in first PCR.

Tumors harboring p53 mutations were identified by SSCP. Individual DNA fragments with shifted mobility were identified as p53 exon 9 with mutation, as compared with the control [Figure 2].

Therefore, p53 mutation status was determined for 2 cases (6.7%). The two samples which showed abnormal SSCP pattern were from separate patients who were classified as p53-positive. However, no mutations were detected in 28 cases (93.3%) classified as p53-negative. As it was mentioned, SSCP was repeated three times to confirm the results. And both of the mutations found were identified in all three times.

Of the two mutations, one was in men and the other belonged to women group. In Figure 2, the SSCP gel shows the nested PCR product, and has revealed the presence of various bands with minimal background contamination.

Correlation between p53 mutations and clinico-pathological parameters

Analyzing pathological grades and tumor stages indicated that the studied TCC cases were distributed as follows: 3 patients with grade I, 21 patients with grade II, 4 patients with grade III, and

2 patients with grade IV of tumor (Invasive type). Women had the same p53-positive bladder cancer as men. Both p53-positive cases were in grade II. In both cases, muscular propia were not involved. However, no significant differences were observed among any clinico-pathological parameters and p53 mutation in Kermanshah people. Thus, there was no indication that prevalence of mutations was associated to grade of tumor. The relationships between grades and sample mutations are shown in Table 3.

DISCUSSION

Most of studies on genome molecules are about p53, and its protein as p53 tumor suppressor is mutant in more than 50% of human cancers.^[19]

P53 alteration (usually missense mutation) occurs in most of human cancers.^[20,21] For example, in 70% of lung cancers, 60% of colon cancers and bladder cancers, head and neck and ovary cancer, p53 mutations are so common. This kind of mutation regularly produces mutant p53, which has a long half life. Consequently, a large amount of this abnormal protein accumulates in nucleus of transformed cells.^[11,22]

Although p53 mutation frequently exists in bladder tumor,^[23-25] it is not clear that in which stage the mutation happens.^[23]

In current study, p53 mutation in bladder cancer patients from Kermanshah was studied. By means of PCR-SSCP, shifted bands were identified in 2 paraffin-embedded bladder samples (6.7%) out of 30 patients with TCC. So, we can announce that the frequency of p53 mutation is 6.7%. This result shows a lower rate of occurrence than other studies about this cancer. The basis of such dissimilarity in different articles, especially when variation is seen in one particular population, may be as follows.

Difference in methodology is one of the reasons. For instance, a number of researchers use IHC, some apply PCR-based molecular methods such as sequencing, SSCP, and others employ a combination of these techniques. The sensitivity of these methods is not similar. For example, IHC (which is in relation to protein expression) in comparison to DNA sequencing has the sensitivity of 72% and specificity of 92%. However, SSCP depending on its condition has the sensitivity of 75-98%. This means that it can recognize 70-98% of point mutations in DNA transcript.^[26] Yet, PCR has its own conditions: For example, when DNA is extracted from paraffin-embedded tissues, primers should have less than 300 bp. because the DNA would be degraded in such circumstance and amplification won't be successful.^[27] If we have poor amplification in PCR, it can make false-positive or false-negative answer, because we have to add more samples in SSCP polyacrylamide gel in this situation, and this can alter DNA movement through the gel. It is suggested that in PCR-SSCP research, a highly resolving agarose gel (4%) be used. The SSCP condition is very significant as well. Firstly, the best DNA product size for SSCP is 150-250 bp. Secondly, electrophoresis

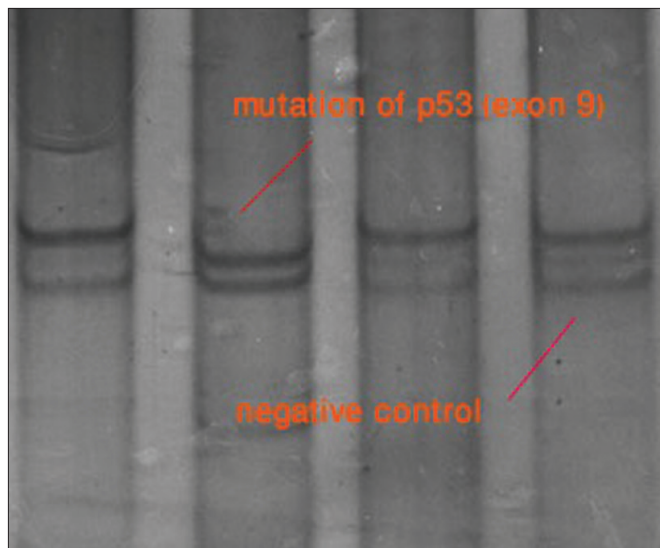


Figure 2: Polymerase chain reaction-single-strand conformation polymorphism autoradiograph showing a 157-bp fragment of p53 exon9 in bladder tumors. Mutant lanes are indicated by an arrow in column 2. Negative control and two other patients without mutation are illustrated in column 4, 1, and 3 respectively

temperature is an important factor that can be 8°C, 10°C, 15°C, 20°C. 4°C has been the best choice in most articles because it demonstrates the most sensitivity and we use this temperature and also cold room to have the least fluctuation in warmth. However, some kinds of mutation may not be recognized in 4°C, thus it's better to repeat the test in 25°C as well.^[28]

Some articles investigated exon 5-8 of p53 in which has more than 90% of mutations.^[29] We choose exon 9, in which less research has been investigated. Other articles have also reported mutation in this exon as well.^[30-32] Consequently, our results along with other articles, which have found p53 mutation in bladder cancer, demonstrate an essential role for p53 in bladder cancer.

To assess correlation between p53 mutations and clinico-pathological parameters, we can say that both p53-positive cases were in grade II (9.5%), but there were no significant levels of discordance between the two approaches. Furthermore, in both cases, muscular propia were not involved. In other clinico-pathological parameters, such as lymph node status, tumor size, and sex, also no significant differences were observed.

Yet, this lack of correlation could be the result of the low number of patients studied.

This is one of the limitations of our study. The other limitation was that, due to the time restriction of the student thesis, we could not send the mutant DNA for sequencing. However, the data presented here are the first report on the p53 abnormalities in bladder cancer patients from Kermanshah. Therefore, we can suppose that the percentage of p53 exon 9 mutation is low in bladder cancer patients from western Iran. However, to characterize the p53 gene alterations and to elucidate its mutation spectrum among Iranian bladder cancer patients, we suggest further studies on a larger number of cases if possible.

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