Mutations of *RAS* Gene Family in Specimens of Bladder Cancer

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Introduction: Studies have shown different types of *RAS* mutations in human bladder tumors with a wide range of mutation frequencies in different patient populations. This study aimed to assess the frequency of specific-point mutations in the *RAS* gene family of a group of Iranian patients with bladder cancer.

Methods: We examined the tumor specimens of 35 consecutive patients with transitional cell carcinoma. The DNA samples were evaluated for the occurrence of *HRAS*, *KRAS*, and *NRAS* activation using a polymerase chain reaction-restriction fragment length polymorphism technique.

Results: None of the patients had mutations in the *RAS* gene family "hot spots" including codons 12, 13, and 61.

Conclusion: We failed to find *RAS* mutations in our bladder tumor samples. These observations may reflect the involvement of different etiological factors in the induction of bladder tumor of which *RAS* mutation might not be present in all populations.

Keywords: bladder neoplasms, oncogenes, RAS genes, codon, polymerase chain reaction, restriction fragment length polymorphism

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INTRODUCTION

Bladder cancer is responsible for the death of 130 000 people annually worldwide.[1] Studies performed by the American Cancer Society in 2000 estimated that 63 210 new cases of bladder cancer would be found in the United States during 2005, and about 13 180 people would die of the disease.[2] Statistical analyses also showed that early detection of bladder cancer could increase the chance of survival.[3] At the molecular level, the *RAS* activating mutations were first discovered in T24 cell line of bladder cancer.[4] The *RAS* gene family consisting of 3 functional genes, *Harvey RAS (HRAS)*, *Kristen RAS (KRAS)*, and *neuroblastoma RAS (NRAS)*, encode highly similar and conserved proteins with a molecular weight of 21 kDa (p21).[5] This protein is localized in the internal part of the cell membrane and has GTPase activity. Mutations in the hot-spot codons 12, 13 (exon1), or 61 (exon2) cause specific amino acid substitutions and result in the loss of GTPase activity.[6] It is well documented that guanosine triphosphates are molecular switches in signal transduction, and different kinds of extracellular signals stimulate conversion of RAS-GDP to RAS-GTP active conformation. The main function of the RAS protein is to induce activation of downstream kinase cascades that results in continuous mitogenic signaling and transformation of immortalized cells.[7]

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Many studies have detected different types of *RAS* mutations in human bladder tumors.[8-14] The results from these studies show a wide range of mutation frequencies. It is not clear whether these differences are related to the different life styles of the studied populations, exposure to different suspected environmental carcinogens, or to the sensitivity of ecogenetic relationships. According to these observations, the present study was aimed to investigate the frequency of specific point mutations of the *RAS* gene family in a group of Iranian patients suffering from bladder cancer.

MATERIALS AND METHODS

Sample Collection

Surgical specimens from 35 patients with histologically confirmed transitional cell carcinoma were collected and stored at -74°C. The patients were consecutively selected from among admitted patients to Sina Hospital. Age, sex, and smoking history of the patients were obtained from their hospital records.

DNA Extraction

Genomic DNA was extracted from the tumoral tissues using proteinase K and phenol extraction methods, and then, it was stored at 4°C.[15]

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

Matched and mismatched oligonucleotide primers were designed or selected from previous studies for amplifying sequences around codon 12 of *HRAS*, codons 12 and 13 of *KRAS*, and codon 61 of *NRAS* in order to generate subfragments only from wild-type polymerase chain reaction (PCR)-amplified *RAS* genes (mutant-type destroys the created restriction site).[16] The primer sequences used were as follows:

HRAS: 5'-GACGGAATATAAGCTGGTGG-3'

and 5'-AGGCACGTCTCCCCATCAAT-3'

KRAS: 5'-ACTGAATATAAACTTGTGGTAG TTGGACCT-3'

and 5'-TTCTCCATCAATTACTACTTGCTT CCTGTA-3'

NRAS: 5'-GACATACTGGATACAGCTGGC-3'

and 5'-CCTGTCCTCATGTATTGGTC-3'

The DNA samples were amplified in a total volume of 50 μL of 10 × PCR buffer (5 μL), dNTP mix (10 mM, 1 μL), each primer (0.5 μL with final concentration of 40 pmol each) and Taq polymerase (Fermentas, Burlington, Canada). Amplification of fragments of the studied genes was carried out with a thermal cycler PCR (Geniu system, Boehringer Mannheim, Germany) using the following thermal profile: 95°C for 5 minutes, 32 cycles; *HRAS*: 95°C for 40 seconds, 6°C for 40 seconds, and 72°C for 45 seconds; *KRAS*: 94°C for 40 seconds, 60°C for 40 seconds, and 72°C for 50 seconds; *NRAS*: 94°C for 30 seconds, 60°C for 40 seconds, 72°C for 30 seconds, followed by a final extension at 72°C for 2 minutes.

Enzyme Digestion

Restriction endonucleases MspI, BstNI, HphI, and MscI (Fermentas, Burlington, Canada) digested codons 12 (*HRAS*), 12 and 13 (*KRAS*), and 61 (*NRAS*), respectively. Any mutation at these codons disrupts the restriction site for the related restriction enzyme. Digestion was carried out in a total volume of 30 uL that contained 12 uL of PCR amplicon and 10 IU of restriction endonuclease. Buffers and incubation conditions (overnight at 37°C) were applied as recommended by the manufactures. The digested fragments were electrophoresed on a 6% polyacrylamide gel (1:59 bis-acrylamide for *HRAS* and *KRAS* genes, and 1:19 for *NRAS* gene) in 0.5 × TBE at 200 V for 1 hour and gels were stained in ethidium bromide. In this work, different sizes of DNA fragments of *HRAS*, *NRAS*, and *KRAS* genes (GenBank accession numbers: NM_005343, NM_002524 and NM_004985, respectively) were obtained by means of PCR amplification.

RESULTS

Patients

The mean age of the patients was 65.8 ± 11.8 years (range, 34 to 85 years), and 74.3% of them were older than 60 years. Twenty-nine patients were men (82.8%) and 18 were smokers (51.4%). Analysis of the pathological grades showed that

23 specimens (65.7%) were low grade (2 low malignant potential, 21 low grade, and 12 high grade).

Polymerase Chain Reaction Amplification

The lengths of the *RAS* amplified fragments according to the designed primers were 420 bp, 65 bp, 144 bp for *HRAS*, *NRAS*, and *KRAS* genes, respectively.

Restriction Enzyme Digestion of HRAS Codon 12

To determine any point mutation at codon 12 of *HRAS*, the restriction enzyme MspI was used. Only the wild-type amplicon containing the endonuclease recognition site could be cut off and give rise to 390-bp and 30-bp fragments. No point mutation on codon 12 of *HRAS* was found (Figure 1).

Restriction Enzyme Digestion of NRAS Codon 61

The restriction enzyme MscI was used for digestion of the codon 61 of *NRAS*. The proper cutting site (*TGG↓CCA)* was created with the help of the forward primer, which led to a single nucleotide change just before codon 61. In case of any mutation, the restriction enzyme MscI would be unable to cut the PCR fragment to 21-bp and 44-bp oligonucleotides (Figure 2).

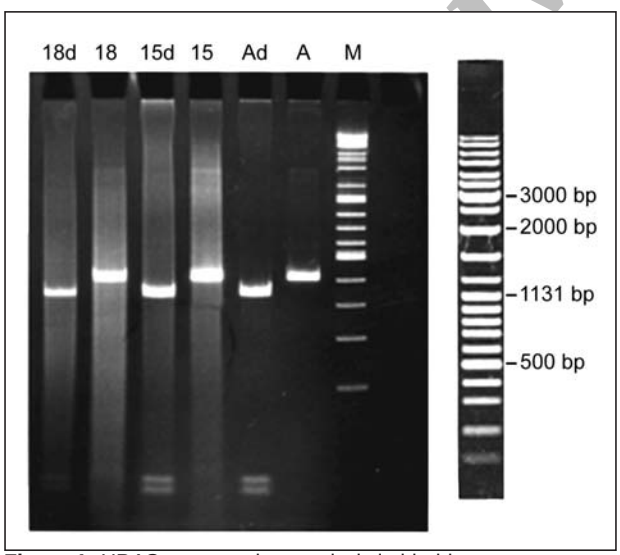

Figure 1. *HRAS* gene product analysis in bladder cancer by polymerase chain reaction-restriction fragment length polymorphism. A, undigested DNA from a healthy person (420-bp); Ad, MspI-cut polymerase chain reaction product of the healthy person (390-bp and 30-bp); Lanes 15 and 18, undigested DNA of patients 15 and 18, respectively; and Lane 15d and 18d, digested products of patients 15 and 18, respectively.

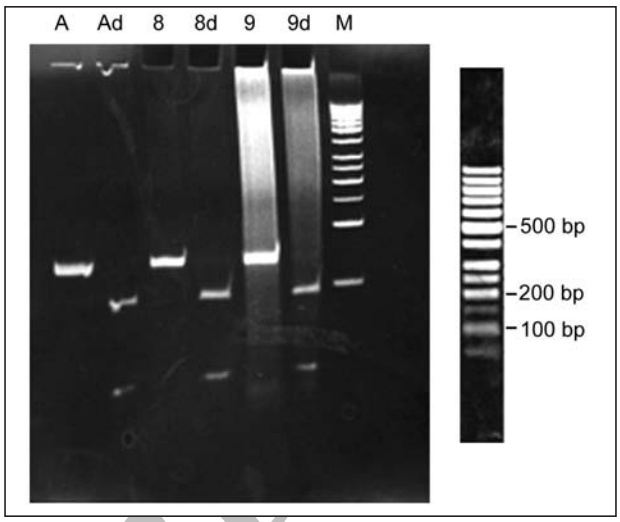

Figure 2. *NRAS* gene product analysis in bladder cancer by polymerase chain reaction-restriction fragment length polymorphism. A, undigested DNA from a healthy person (65-bp); Ad, MscI-cut polymerase chain reaction product of the healthy person (44-bp and 21-bp); Lane 8 and 9, undigested DNA of patients 8 and 9, respectively; and Lane 8d and 9d, digested products of patients 8 and 9, respectively.

Restriction Enzyme Digestion of KRAS Codons 12 and 13

The restriction enzyme BstNI was used for codon 12 of *KRAS* gene digestion. A primer was designed, so that the cutting site was created just before codon 12. Only the wild-type *KRAS* PCR product would be cut by BstN1, yielding 2 fragments around 115-bp and 29-bp oligonucleotides. For codon 13, *GGTGA7/8↓* is the recognition site for HphI and is cut off by the enzyme. This site does not exist naturally, but it would appear in any type of mutation. Digestion reaction was carried out for each sample and no mutation was detected for *KRAS* (Figure 3).

DISCUSSION

Studies on a variety of tumors have demonstrated some "hot spots" in *RAS* gene family that are susceptible to point mutations. The frequent mutations are changes of glycine to valine at codon 12, glycine to cysteine at codon 13, and glutamine to arginine/lysine/leucine at codon 61.[17] The incidence of *RAS* mutation varies and is

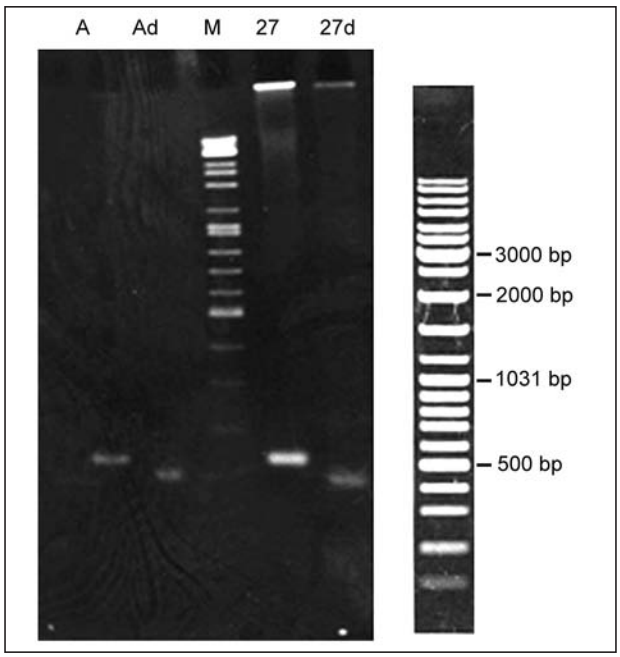

Figure 3. *KRAS* gene product analysis in bladder cancer by polymerase chain reaction-restriction fragment length polymorphism. A, undigested DNA from a healthy person (144-bp); Ad, BstNI-cut polymerase chain reaction product of the healthy person (115-bp and 29-bp); Lane 27, undigested DNA from patient 27; and Lane 27d, the digested product from patient 27.

greatly dependent on the tissue or cell type from which the cancer cells are derived. Although *RAS* mutations occur in 75% to 95% of pancreatic carcinomas and 50% of colon carcinomas, they are rare in several other neoplasms.[18-20] The *HRAS* mutation was first detected in the human bladder cancer cell line T24.[7] Subsequent studies demonstrated that *HRAS* mutations were more frequently observed in urinary tract tumors than the *KRAS* or *NRAS* genes.[21] This initial expectation has been materialized, since later analysis of uncultured bladder tumors showed that only about 10% of the samples contained a mutated *HRAS*.[22-24] However, later reports showed higher frequencies. While Fitzgerald and associates reported mutations in the *HRAS* gene in 44% of urine sediments from bladder cancer patients,[9] Czerniak and coworkers observed *HRAS* mutation specifically in connection to codon 12 in 45% of the bladder cancers.[10] Also, in a recent study by Jebar and colleagues on 98 bladder tumors and 31 bladder cell lines, *RAS* mutation was detected in 13% of both types of samples.[11] In total, there were 10 mutations in *HRAS*, 4 in *KRAS*, and 4 in *NRAS*. On the other hand, various levels of *RAS* mutation at codon 12 have recently been reported in bladder cancer. While Zhu and associates and Buyru and coworkers showed 46.7% and 39% point mutation of *HRAS* at codon 12, respectively,[3,12] Cattan and associates detected only 1% of such alterations.[13] Furthermore, Przybojewska and colleagues found the *HRAS* mutation in 84% of patients with bladder cancer using a PCR-restriction fragment length polymorphism assay.[16]

In contrast to the above discussed investigations showing *RAS* activation, our study detected no mutation in the *RAS* gene family in any grades of bladder cancer in the 35 studied patients. It should be mentioned that the RAS protein dysfunction may occur not only as a result of mutations in the *RAS* gene, but also due to changes in the protein level. Quantitative alterations in the expression due to gene amplification or overexpression could lead to continuous proliferative signals needed for cell propagation. Previous studies demonstrated increased expression of RAS protein in carcinoma in situ and high-grade tumors, but not in hyperplasia or low-grade tumors when immunohistochemical technique was applied.[14] Vageli and associates reported an increase in *RAS* transcripts in about 40% of the bladder cancers, as well.[25] All these studies indicate that the precise frequency of *RAS* mutations in human bladder cancer is still unclear. The observed discrepancies in the mutation pattern of *RAS* gene family among different populations suffering from bladder cancer may either reflect different etiological mechanisms involved in disease progression or alternative *RAS* dysfunction such as gene amplification and/or overexpression.[14,25] Notably, it is not surprising that Iranian patients have a specific mutation pattern for *P53* gene as it has been reported for esophageal cancer.[26] Ecogenetic relationships and cultural conditions of may somehow explain the absence of *RAS* gene family mutation in our patients. Although the results so far reported still remain controversial, activation of the *RAS* oncogene by point mutation or overexpression may be important in the carcinogenesis and progression of human bladder cancer.

Smoking is an established risk factor for bladder

cancer.[27] Consistent with the epidemiological evidence for an association between bladder cancer and smoking, we found that about 51% of our patients were smokers, which shows a direct correlation between smoking and the incidence of bladder cancer. However, the group under our investigation is too small in number to be considered for epidemiological conclusions. The other related risk factor of bladder cancer is age. Our data showed nearly three-fourth of our patients were over 60 years of age. This is in accordance with the previous data showing more than 65% of bladder cancer patients in the United States were older than 65 years.[28]

CONCLUSION

We failed to find *RAS* gene mutation in our patients with bladder tumors. This observation may reflect the involvement of different etiological factors in the induction of this tumor. Due to the reported studies and possibility of the involvement of various etiological factors, it is interesting to study the situation among Iranian patients suffering from bladder cancer with various pathological low-grade and high-grade tumors regarding the status of these three genes.

CONFLICT OF INTEREST

None declared.

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CLINICAL TRIAL REGISTRATION

The Iranian Registry of Clinical Trials (IRCT) has been launched (http://www.irct.ir/) thanks to the sponsorship by the Iranian Ministry of Health. We strongly encourage researchers who would like to publish reports of their clinical trial in *Urology Journal* to register their studies in the IRCT or other registries that are proposed by the World Health Organization and the International Committee of Medical Journal Editors.

Registration of clinical trials before starting the research project is now considered a primary requirement by these organizations, and it is also emphasized by the World Medical Association Declaration of Helsinki. This helps to ensure that decisions about healthcare are informed by all of the available evidence, ensure that a trial and its results are publicly disclosed, avoid unnecessary duplication, facilitate recruitment of participants, identify gaps in research, encourage collaboration among researchers, and make it possible to identify potential problems and improve clinical trials.

The links below would help you to find clinical trial registries and useful information on this issue:

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