Genetic Aberrations of the *K-ras* Proto-oncogene in Bladder Cancer in Kashmiri Population

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Purpose: To assess the frequency of specific point mutations in the *K-ras* gene in a group of Kashmiri patients with bladder cancer.

Materials and Methods: We analyzed the incidence of *K-ras* exon 1 gene mutations in tumors and surgical margins in 60 patients with transitional cell carcinoma of varied clinical stages and histological grades using the polymerase chain reaction-single strand conformation polymorphism and DNA sequencing.

Results: A significant correlation was found between the *K-ras*, the lymph node status, and tumor recurrence ($P < 0.05$). Also, smokers and patients with higher tumor grade showed a significantly higher relative risk of developing *K-ras* mutations than the normal ones.

Conclusion: *K-ras* exon 1 gene mutations were found with low frequency in the bladder cancer tumors from Kashmir valley, which suggests that *K-ras* gene might be involved in a sub-set of bladder tumors, but it needs further investigation on a larger cohort sample to authenticate the current findings.

Keywords: proto-oncogene protein, urinary bladder neoplasm, ras genes, genetics

Urol J. 2010;7:168-73.
www.uj.unrc.ir

INTRODUCTION

Bladder cancer, which is the fourth most incident cancer in the USA and affects more than 350 000 subjects worldwide, is one of the leading causes of mortality.[1,2] In Kashmir, the northern region of India, bladder cancer is considered as the 9th most common cancer.[3]

The *ras* gene family consisting of 4 functional genes, *Harvey ras (H-ras)*, *Kristen ras (K-ras) A and B*, and *Neuroblastoma ras (N-ras)*, encode highly similar and conserved proteins with a molecular weight of 21 kDa.[4] These closely related proteins are localized in the internal part of the cell membrane and have intrinsic GTPase activity, which regulates their cellular activity.[5]

The main function of the *ras* proteins is to induce activation of downstream kinases belonging to mitogen-activated protein kinase pathway, which in turn results in continuous mitogenic signaling and transformation of immortalized cells.[6] Because of their active involvement in proliferative and/or differentiative signals within the growing cell, *ras* genes are the most common targets for somatic gain-of-function mutations in almost all human cancers that lead to the formation of constitutively active proteins due to altered intrinsic GTPase activity.[7]

Mutated *ras* genes are associated with 15% to 30% of all human cancers, with highest frequencies

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Received August 2009
Accepted February 2010

associated with pancreatic, lung, and colon carcinomas.[8,9] These mutated *ras* genes encode constitutively active proteins, most commonly with single amino acid substitutions at residues 12, 13, or 61 causing usually the loss of intrinsic GTPase activity of the proteins.[10] The first report of the activating *ras* mutations in bladder tumors was made by Bos in *T24 cell line*.[11] Since then, many studies have been carried out on human bladder tumors across the globe, and reports of different types of activating *ras* mutations involved in the tumorigenesis are well documented in the available literature.[12-19]

Because of these observations and the possibility of activating *ras* mutations to be involved in the tumorigenesis of bladder cancer as well as various suspected etiological factors, the present study was carried out in ethnic Kashmiri population to investigate the frequency of specific point mutations in the *K-ras* gene and to correlate them with the various etiological parameters to which our population is exposed.

MATERIALS AND METHODS

Sample Collection

Surgically resected specimens of 60 patients were collected from Department of Urology, Sher-I-Kashmir Institute of Medical Sciences, Kashmir, India. The study protocol was approved by the Research Ethics Committee of Sher-I-Kashmir Institute of Medical Sciences. Informed consent was obtained from each patient and/or guardian on pre-designed questionnaire (Available on request). Data regarding age, sex, and smoking history of the patients were obtained. Patients who had received previous chemotherapy for a metastatic disease were excluded.

In order to avoid evaluator variability, resected tissue specimens were brought fresh from the theater to Department of Pathology, where they were meticulously examined by two independent and experienced pathologists. The excision of the tumor was histologically proven by examination of the resected margins. All tumors were histologically confirmed to be transitional cell carcinoma. The specimens (both tumor and adjacent normal tissues) were snap-frozen at -70°C immediately until further analysis. Patients were followed up for 1 year after the surgical resection of the tumor.

DNA Extraction

We extracted DNA from primary tumors and adjacent noncancerous tissues, using the DNA Extraction Kit II (Zymo Research), for examining mutations in the *K-ras* exon 1 gene.

Polymerase Chain Reaction-Single Strand Conformation Polymorphism (PCR-SSCP)

Exon 1 of the *K-ras* (containing hot spot codons 12 and 13) was amplified using the previously described specific primers.[20] Polymerase chain reaction was performed in a 25 μl volume containing 50 ng of genomic DNA, 1 × PCR buffer containing 15 mM $MgCl_2$, 100 μM each of dATP, dGTP, dTTP, dCTP, 1.5 unit of *Taq* DNA polymerase (Biotools, Spain), and 10 μM of forward and reverse primers. The amplification program was as initial denaturation at 95°C for 5 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 48°C for 30 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 7 minutes. In every instance, positive human genomic DNA (Genei, India) was also amplified as internal control.

Polymerase chain reaction products were run on 2% agarose gel and analyzed under a ultra violet illuminator (Figure 1). The single strand conformation polymorphism analysis of the amplicons of exon 1 of *K-ras* was performed on 6% non-denaturing polyacrylamide gel electrophoresis utilizing either non-radioactive silver staining or radioactive procedures.[20] The purified PCR amplicons of the tumor samples showing mobility shift on SSCP analysis (Figure 2) and randomly chosen normal samples were used for direct DNA sequencing (Figure 3), using the automated DNA sequencer ABI prism 310.

Statistics

Fisher's exact test (one-tailed) was used to evaluate the association between clinicopathological variables in case of *K-ras*. *P* values less than .05 were considered statistically significant.

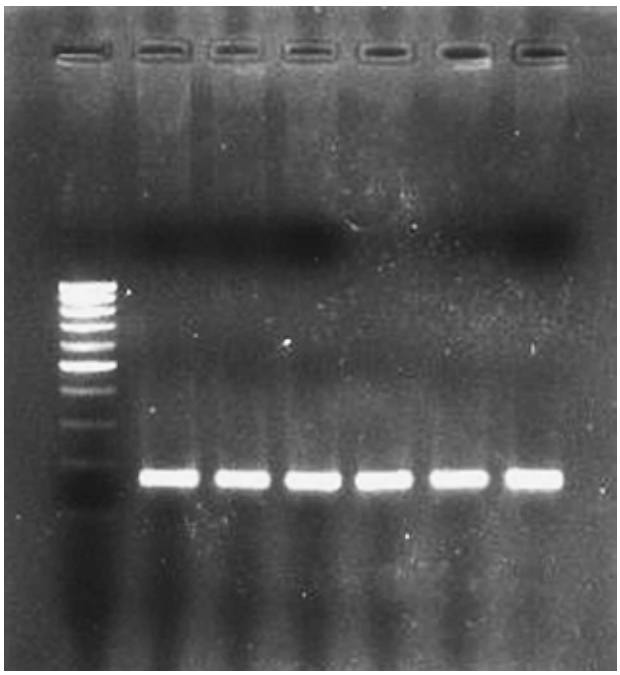

Figure 1. Amplified DNA fragments of exon 1 of *K-ras* (162 bp amplicon) gene; First Lane represents 100 bp molecular ladder and rest represent amplicons from different tumor tissues.

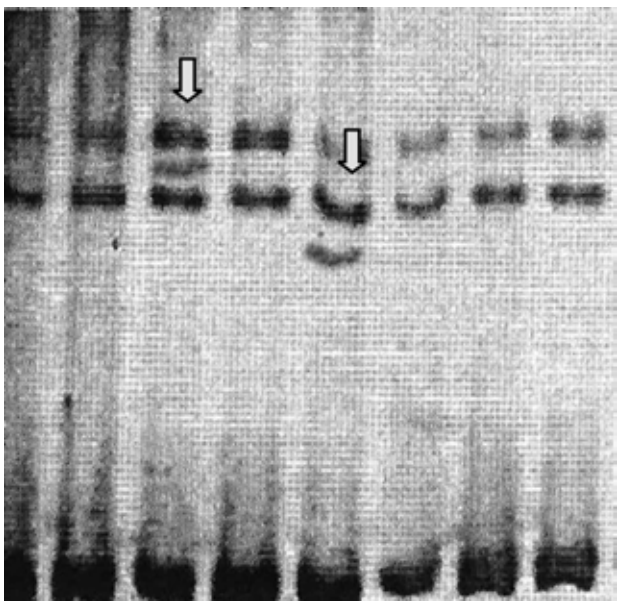

Figure 2. A radioactive SSCP analysis of *K-ras* exon 1 showing mobility shifts in tumor sample.

RESULTS

The mean age of the patients was 61 years and 63.3% of the patients were older than 50 years. About 83.4% were men, 80% were rural, and 75% were smoker (Table 1).

The mutational examination of exon 1 of *K-ras* gene revealed an overall *K-ras* mutation in 7 subjects aggregating to about 11.67% of the studied sample, which included 6 transitions and 1 transversion. Transition mutation was of only one type G→A, and sole transversion was G→C. Further more, of a total of 7 mutations, 4 affected codon 12 and 3 affected codon 13 (Table 2). Two cases were G12D (GGT > GAT), 2 cases were G13D (GGC > GAC), 2 cases were G12S (GGT > AGT), and 1 case was G13R (GGC > CGC). The mutations in exon 1 of *K-ras* gene were found relatively more at codon 12 (57.1%) than codon 13 (Table 2), which is consistent with the already available data.[21] No germ line mutations were found, indicating that in every case the change was somatic.

Statistical analysis of the mutants with respect to various clinicopathological variables revealed a significant association ($P < .05$) between the *K-ras* mutation, the lymph node status, and tumor recurrence. Furthermore, smokers and patients with higher tumor grade showed a significantly higher relative risk of developing a bladder cancer than the normal ones (OR > 2) (Table 1).

DISCUSSION

A number of different studies carried out on various different cancers have demonstrated some hot spot regions in *ras* gene family that are susceptible to point mutations, the frequent among them are changes of glycine to valine/aspartate/serine at codon 12, glycine to arginine/cysteine at codon 13, and glutamine to arginine/lysine/leucine at codon 61.[21,22]

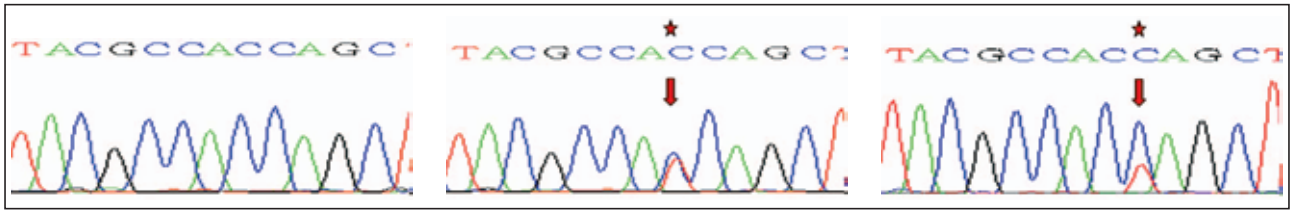

Figure 3. Partial nucleotide sequences (reverse) of the normal and mutants – T3, T46 in exon 1 of *Kirsten ras* oncogene.

Table 1. Clinico-epidemiological variables of the patients with bladder cancer versus the mutant phenotypes of the *K-ras* exon 1 gene

Variable	Total (N = 60) (%)	Mutants (M = 7) (%)	*P*; OR; CI (95%)
Sex	Males: 50 (83.4) Females: 10 (16.6)	Males: 6/50 (12.0) Females: 1/10 (10.0)	.67, 1.2273; 0.1313 - 11.4727
Age	≤50: 22 (36.7) >50: 38 (63.3)	≤50: 3/22 (13.6) >50: 4/38 (10.5)	.50, 1.3421, 0.2713 - 6.6394
Dwelling	Rural: 48 (80) Urban: 12 (20)	Rural: 5/48 (10.4) Urban: 2/12 (16.7)	.42, 0.5814; 0.0982 - 3.442
Smoking status	Smokers: 45 (75) Nonsmokers: 15 (25)	Smokers: 6/45 (13.3) Nonsmokers: 1/15 (6.7)	.43; 2.1538; 0.2379 - 19.5038
Differentiation grade	II: 21 (35) III +IV: 39 (65)	II: 4/21 (19.1) III + IV: 3/39 (7.7)	.18; 2.8235; 0.5676 - 14.0446
Histological type	S: 35 (58.3) MI: 25 (41.7)	S: 3/35 (8.5) MI: 4/25 (16.0)	.31; 0.4922; 0.0999 - 2.4256
Lymph node status	NO: 55 (91.7) YES: 5 (8.3)	NO: 4/55 (7.3) YES: 3/5 (60.0)	.009; 0.0523; 0.0067 - 0.4096
Tumor Recurrence	NR: 47 (78.3) R: 13 (21.7)	NR: 2/47 (4.3) R: 5/13 (38.5)	.003; 0.0711; 0.0117 - 0.432
Stage	PT1: 39 (65) PT2: 21 (35)	PT1: 4/39 (10.3) PT2: 3/21 (14.3)	.46; 0.6857; 0.1383 - 3.4007

Table 2. Details and nature of *K-RAS* exon 1 gene mutations in patients with bladder cancer from Kashmir valley

Patient ID	Age / Sex	Rural/ Urban	Histopathological Stage	Histopathological Grade	Smoking Status	Type	Codon Number	Base Change	Amino acid Change
T3	80/M	R	PT1	II	Ever	MI	12	GGT>GAT	Gly>Asp
T11	45/M	R	PT2	III	Ever	S	12	GGT>AGT	Gly>Ser
T17	60/M	R	PT1	II	Ever	MI	12	GGT>GAT	Gly>Asp
T22	81/F	U	PT1	IV	Ever	MI	13	GGC>CGC	Gly>Arg
T42	65/M	R	PT2	II	Ever	MI	13	GGC>GAC	Gly > Asp
T46	41/M	R	PT1	II	Never	S	12	GGT>AGT	Gly>Ser
T55	38/M	U	PT2	IV	Ever	S	13	GGC>GAC	Gly > Asp

M, indicates male; F, female; R, rural; U, urban; MI, muscle invasive; S, superficial; and Base change, mutated or inserted nucleotide underlined.

The incidence of *ras* mutation varies and is 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.[23-25] Mutations at codon 12 of *K-ras* are infrequent in the bladder cancer.[26,27] In a recent study, Jebar and colleagues have found *K-ras* mutations in 3 of 98 patients with the bladder cancer.[16] Interestingly, experimental studies on transgenic mice have shown that tissue-specific expression of a *K-ras* transgene in the urothelium leads to urothelial hyperplasia and superficial papillary tumors.[28] These observations suggest that activation of *ras* may contribute to early steps of carcinogenesis in the bladder.[16,26-28]

Results of the present investigation confirmed the role of *K-ras* mutations in the development of urinary bladder carcinoma, as we found 11.67% tumors (7/60 tumors) having mutations in this gene in Kashmiri population, which is in consistent with many of the previous studies on bladder cancer.[12,16,27] Furthermore, in this study, we also found a significant correlation between the *K-ras* mutant status and the lymph node involvement and tumor recurrence, suggesting a possible role of *K-ras* proteins in the metastasis of the cancer. The activating mutations of *ras* proteins have been previously implicated in all aspects of the malignant tumor, especially cellular proliferation, transformation, invasion as well as metastasis.[29] Furthermore, Campbell and Der[30] demonstrated that activation of *ras* proteins causes an increase in the transformative, invasive, and metastatic properties of the murine fibroblast cells.[31] Other studies also showed the same concomitant results.[32,33] Since the transformation to a metastatic phenotype requires many changes in cell-cell adhesion, Yan and associates have

reported that mutant *K-ras*, but not *H-ras*, causes disruption of the adhesive qualities of the mutant cell due to oncogene's ability to interfere with the maturation of cell surface integrins.[34]

Furthermore, in our study, a significantly higher relative risk of *K-ras* mutant status was observed in patients with history of smoking and/or having higher tumor grade than the normal ones (OR > 2) (Table 1). This observation is in harmony with the other studies, where smoking has been implicated as one of the risk factors of the bladder cancer.[35] In our study, almost 75% of the patients with the bladder cancer were smoker and they were almost two times more prone to *K-ras* mutations than the normal ones (OR = 2.15).

The *K-ras* activating mutations that were identified in this study were missense in nature, 57.15% were found at codon 12 and 42.85% at codon 13; these results were consistent with other reported studies.[12,20] The mutations of codons 12 and 13 are the most common genetic aberration in *K-ras*, involving the substitution of the active site of glycine residue, which in turn changes the functionality of *K-ras* protein.[20] The substitution at codons 12 and 13 can either alter intrinsic GTPase activity of *K-ras* protein or change its ability to interact with wide variety of regulators.[33] The effectiveness of the substitution depends on the amino acid that replaces the original glycine (at 12/13). In this study, we also found that all *K-ras* mutants were G>R or G>S or G>D variants. It has been reported that replacement of glycine residues with valine or arginine has the aggressive transforming capability followed closely by serine and glutamine variants.[7,21,36,37] All of the above variants have the diminished GTPase activity due to which they remain activated under basal conditions, too, as there is no switching back to inactive GDP-bound form.[7] Substitution by proline makes *K-ras* protein resistant to GAP activity and hence is less aggressive in phenotypic expression.[37]

Of a total of 7 missense mutations, 6 (85.7%) were transitions and only 1 (14.3%) was transversion. All transistions were A>G type, 4 affecting codon 12 and 3 affecting codon 13. The sole transversion of G>C occurred at codon 13 causing replacement of glycine to arginine. The G>A transitions have been already reported to be the most common genetic change in *K-ras* and incidentally these aberration also score second in the human gene mutation database after C>T conversion.[38]

CONCLUSION

To sum up, we can say that *K-ras* exon 1 gene mutations were found with low frequency in bladder cancer tumors from Kashmir valley, which suggests that *K-ras* gene is involved in a sub-set of bladder tumors. Nevertheless, these observations need further investigations in a bigger cross section of the patients with bladder cancer and relevant controls.

CONFLICT OF INTEREST

None declared.

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