

RESEARCH COMMUNICATION

Screening of N-ras Gene Mutations in Urothelial Cell Carcinomas of the Urinary Bladder in the Kashmiri Population

Arshad A Pandith¹, Zaffar A Shah¹, Nighat P Khan², Adfar Y Bhat², Saleem M Wani³, Mushtaq A Siddiqi^{1*}

Abstract

Background and Aims: The objective of this study was to assess the frequency of specific-point mutations in N-ras of the RAS gene family in a group of Kashmiri patients with bladder cancer and to observe any association with clinicopathological parameters. **Methods:** Paired tumor and normal tissue specimens of 55 consecutive patients with urothelial cell carcinoma were screened and DNA was extracted for detection of N-ras activating mutations in exons 1 and 2. In addition, blood was also collected from all the cases to rule out any germ line mutation. **Results:** Specific point mutations of activated N-ras were detected in 9% (5 of 55) of the bladder cancer patients, all being missense. The base substitutions identified included three transversions (two G to T and one A to T) and two transitions (A-G). Sixty % of the mutations were detected in codon 61 and 40% in codon 12. No significant correlations were found between the mutations and clinical features. **Conclusion:** Although N-ras gene mutation might be one of the mechanisms underlying oncogenesis of urothelial cancer, it seems to be a relatively rare event in Kashmiris, pointing to involvement of different etiological factors in the induction of bladder tumor in this population

Key Words: Urothelial cell cancer - N-ras - missense mutations - transversions

Asian Pacific J Cancer Prev, 10, 1063-1066

Introduction

Urinary bladder (or bladder) cancer is one of the most common cancers worldwide, with the highest incidences in industrialized countries (Pelucchi et al 2006). It is the fourth most incident cancer in males and ninth most in females accounting for more than 67,000 new cases per year in United States (Jemal et al 2007), where the annual age adjusted incidence rate for men is approximately 32 per 100,000 (Jemal et al 2005).

Urothelium cancers account for 5.6% of male and 1.8% of female cancers in India with an actual crude rate (ACR) incidence of about 1 in 174 in men and 1 in 561 in women (Kamarana et al., 2000). From an epidemiological survey (Dhar et al 1993) in Kashmir, bladder cancer has an annual incidence of 9.7 (2.5%) ranking 9th in all types of cancers. This was a partial presentation of bladder cancer incidence as the survey was done in a single hospital catering the needs of 1/3 of the population. Presently bladder cancer is showing an alarming increase in Kashmir as evidenced by the fact that we could record and collect samples from 55 patients from early March 2008-2009.

Histologically, more than 90% of bladder-cancer cases are transitional cell (urothelial) carcinoma, approximately 5% are squamous cell carcinoma, and less than 2% are adenocarcinoma. Nearly 80% of patients who initially

present with bladder TCC have tumors confined to the mucosa or sub mucosa—so-called superficial “non-muscle-invasive” bladder cancers (Dinney et al., 2004).

Classically, bladder cancer has been associated with exogenous and environmental risk factors. The 2 best known risk factors for bladder cancer are smoking and occupational exposure. Compared with the general population, smokers are at 2 to 4 times greater risk of bladder cancer and heavy smokers are at 5 times the risk (Henney et al., 1992). The incidence of bladder cancer was strongly associated with occupational exposure to aromatic amines used in the dye industry, before their potent carcinogenicity to the bladder was demonstrated (Zeegers et al., 2004).

The RAS genes (NRAS, KRAS, and HRAS) encode 21-kDa proteins that are members of the super family of small GTP-binding proteins, which have diverse intracellular signaling functions including control of cell proliferation, growth, and apoptosis (Clavel et al., 2007). Somatic activating mutations in RAS are present in up to 30% of all human cancers (Malumbres et al., 2007). Previous studies have detected N-ras oncogene not only in a neuroblastoma (Barbacid et al., 1987) and sarcoma (Shimizu et al., 1983) cell lines but frequently in human hematopoietic tumors as well (Joaõ et al., 2007).

Ras mutations found in cancer cells introduce amino

¹Department of Immunology and Molecular Medicine, ²Department of Biochemistry, ³Department of Urology, Sher-I-Kashmir Institute of Medical Sciences, Srinagar, Kashmir, India *For Correspondence: vc.tmuk@gmail.com, arshaajiz@gmail.com

acid substitutions at positions 12, 13 and 61. These changes impair the intrinsic GTPase activity and confer resistance to GAPs, thereby causing cancer-associated mutant Ras proteins to accumulate in the active, GTP bound conformation (Mitin et al., 2003). NRAS and HRAS mutations predominate in melanoma and bladder cancer (Bos et al., 1989; Downward et al., 2005).

Subjects and Methods

Patients and controls

Fifty-five consecutive samples of the patients who underwent TURBT and radical cystectomy in surgery department at the Sher-i-Kashmir institute Of Medical Sciences 2008–2009 were enrolled into this study. Median age at time of diagnosis was 59 years (range, 38–80); male: female ratio was 6:1. Forty-eight patients were men (87%) and 43 (78%) were smokers. Almost all the patients had attended the hospital with a clinical presentation of haematuria. Grading of cancers was performed according to the WHO classification and staging according to the 1997 TNM classification guidelines (UICC 1997).

Diagnostic slides prepared from fresh tumor samples were reviewed by a panel of two expert pathologists to confirm diagnosis and ensure uniformity of classification criteria. All the samples were confirmed to be histological bladder cancers. Information on grade was available on 53 and on stage for 55 samples. There were four grade-1 tumors, 18 grade-2, 20 grade-3, 10 grade-4 and one was intermediate grade (Table 1). In total 21 were pTa, 16 pT1 and 17 pT2 while as one sample was carcinoma in situ. Samples of 5 ml venous blood from each patient were collected in EDTA as controls.

DNA extraction

Tumor samples (both tumor and adjacent normal) collected after TURBT were snap-frozen immediately and stored at -70°C. DNA from neoplastic tissue was extracted using DNeasy Tissue kit (Qiagen GmbH, Hilden-Germany) according to the manufacturers enclosed protocol.

Polymerase Chain Reaction (PCR)

Exon 1 and 2 of the N-ras containing hotspot codons 12 and 13, and 61 were amplified using previously described specific primers as NRAS exon 1F GACTGAGTACAACTGGTGGTGG NRAS exon 1R GGGCCTCACCTCTATGGTG. NRAS exon, 2F; GGTGAAACCTGTTTGTGGGA NRAS exon 2R ATACACAGAGGAAGCCTTCG.

The PCR products were run on 2% agarose gel and analyzed under a UV illuminator. The Single-Strand Conformation Polymorphism (SSCP) analysis of the amplicon of exon 1 and 2 of N-ras was performed on 6% non denaturing polyacrylamide gel (PAGE) utilizing either non-radioactive silver staining or radioactive procedures (Bosari et al 1995). The purified PCR amplicons of the tumor samples showing mobility shift on SSCP analysis and randomly chosen normal samples were used for direct DNA sequencing, using the automated DNA sequencer ABI prism 310.

Table 1. Details and Nature of N-ras Exon 1& 2 Mutations in Bladder Cancer Patients from Kashmir Valley

Age/ Sex	R/U	Histo ¹ Stage	Grade	Smoking Status	Codon Type	Base Change	AA Change
80/M	R	PTa	II	Ever	S 12	GGT>TGT	Gly>Cys
81/F	U	PT1	III	Ever	S 12	GGT>TGT	Gly>Cys
65/M	R	PT2	III	Ever	MI 61	CAA>CTA	Gln>Leu
43/M	R	PT2	III	Never	MI 61	CAA>CGA	Gln>Arg
57/M	U	PT2/3	IV	Ever	MI 61	CAA>CGA	Gln>Arg

¹Histopathological; AA, Amino acid; M, Male; F, Female; R, Rural; U, Urban; MI, Muscle Invasive; S, Superficial

Table 2. Relationship of Specific N-ras 12/61 Mutations with Clinicopathological Variables

Variables	Cases	Wild type	Mutant
Mean age (years)	59	61	62
Sex			
Male	48	44 (87%)	4 (13%)
Female	7	6 (85%)	1 (15%)
Smoking			
Ever	43	39 (90%)	4 (9%)
Never	12	12 (93%)	1 (8%)
Stage			
Ta	21	20 (95%)	1 (5%)
T1	16	15 (93%)	1 (7%)
T2 or higher	17	14 (82%)	2 (18%)
Grade			
1	4	5(100%)	0 (0%)
2	18	17 (94%)	1 (6%)
3	20	18 (90%)	2 (10%)
4	10	8 (80%)	2 (20%)

Results

tDNA samples to be examined for mutant codons were prepared from 55 specimens of bladder cancer tissue (patients 1 to 55), 10 specimens of normal bladder mucosa (patients 56 to 66). DNA fragments that included exon 1 & 2 of the N-ras gene were amplified by PCR which on sequencing revealed an overall N-ras mutation in 06 tumors (9%) in male patients except one mutation in woman.

A total of 55 confirmed transitional (urothelial) cell carcinoma samples for mutations in N-ras gene run by single-strand conformation polymorphism (SSCP) analysis to screen for mutations in exons 1 and 2 and subsequently followed by direct sequencing of the samples showing mobility shift in SSCP. The samples which showed mobility shift in SSCP paralleled with the DNA sequencing which corresponded to five mutations in all (Figures 1, 2a & 2b). Thus the total mutations in exon 1 and 2 of N-ras gene detected in this study were 9% (5/55). There were five mutations (2 transitions and 3 transversions), two were A-G transitions, two G-T transversions, and one A-T transversion, out of which two affected codons 12 (40%) and three affected codon 61 (60%) (Table1). No mutation was detected in codon 13 in this study. The samples harboring mutations had two G61C (GGT>TGT), two Q12R (CAA>CGA), and one Q61L (CAA>CTA) (Table1). The mutations were mostly seen in smokers (4/48 and one mutation was detected in bladder tumor of non smokers). The 5 tumour samples with mutation included, one grade-2 and two grade-3 tumours and two grade-4, but stage wise were one pTa

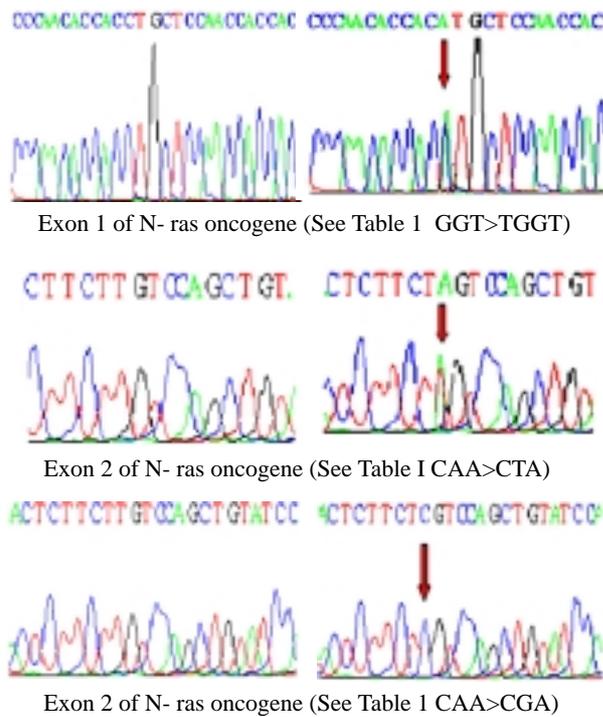


Figure 1. Partial Normal and Mutant Nucleotide Sequences (reverse) (arrows point to mutations)

and one pT1 and two pt2/3 tumours (Table2). Any association with the age, gender, location of tumor, and/or its size was not found. No germ line mutations were found, indicating that in every case the change was somatic.

Discussion

Activating Ras mutations occur in ~30% of human cancers. Specific Ras genes are mutated in different malignancies: Various 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(Levesque et al 1993 and Sameer et al 2009). NRAS and HRAS mutations predominate in melanoma and bladder cancer, respectively (Downward et al 2005 and Bos et al 1989). In most cases, the somatic missense. Ras mutations found in cancer cells introduce amino acid substitutions at positions 12, 13 and 61. These changes impair the intrinsic GTPase activity and confer resistance to GAPs, thereby causing cancer-associated mutant Ras proteins to accumulate in the active, GTPbound conformation (Trahey et al 1987). The ‘hot spot’ mutation at codon 12, 13 or 61 in the N-ras gene has been described in many kinds of human cancers (Tada et al 1990).

Though our study found relatively low frequency of specific N-ras mutations but it confirms the role in the development of urinary bladder carcinoma, as we detected 09% tumors (5/55 tumors) having mutations in this gene in Kashmiri population. Out of these 60% were in 61, 40% in codon 12 and no mutation in codon 13. Five of the mutations detected in males were ever smokers and one mutation was seen in an elderly non smoker female.

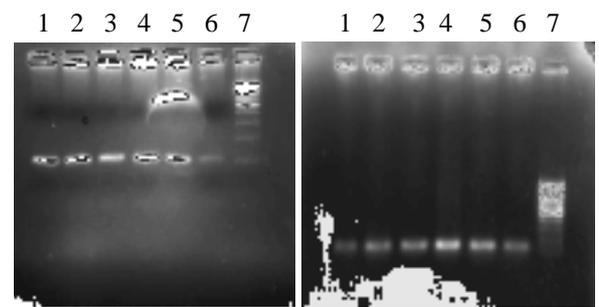


Figure 2. Amplified DNA Fragments. a) Exon 2 of N-ras (103bp amplicon) gene, Lane 7, 100 bp molecular ladder , Lanes 1-6, Amplicons from different tumor tissues; b) Exon 1 of N-ras (118bp amplicon) gene, Lane 7, 100 bp molecular ladder, Lanes 1-6, Amplicons from different tumor tissues

Although diet might also influence bladder carcinogenesis, owing to the many potential carcinogens or chemo preventive nutrients therein, no consistent association between intake of selected nutrients or micronutrients and positive cases has emerged in this study

All of the mutations that were identified in this study were missense with a rate of transversions as 60% and transitions 40%. Three mutations in codon 61 and two in codon 12 were identified and the predominance of these mutations was expected, as most of the mutations found in N-ras in human tumors involve these two codons. In a recent study (Jebar et al 2005) on 98 bladder tumors and 31 bladder cell lines, RAS mutation was detected in 13% of both types of samples . In total, there were 10 mutations in H-ras, 4 in KRAS, and 4 in NRAS. In N-ras mutation only one mutation was found in codon 12 and three in codon 61. Codon 12 & 61 codes for Glycine and glutamine residues, which play an important role in the catalytic site of RAS. In most cases, the somatic missense Ras mutations found in cancer cells introduce amino acid substitutions at positions 12, 13 and 61. The substitutions of 12 and 13 amino-acid residues in RAS alter its GTPase activity to a different extent and/or its ability to interact with its regulators, depending upon the substituted amino-acid residue.

As smoking is an established risk factor for bladder cancer. Consistent with the epidemiological evidence for an association between bladder cancer and smoking, we found that about 78% 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 mutations detected in this study comprised three transversions (two G: C-T: A and one A: T-T: A) and two transitions (A: T-G: C). A similar spectrum of substitutions is characteristic of p53 mutations in bladder tumors (Sidransky et al., 1991).This may reflect the known association of increased bladder cancer risk with cigarette smoking (Hartge et al., 1987). A G-T transversion is not only the most frequently observed type of mutation but has been linked to smoking (Habuchi et al., 1993) found A:T=G:C transitions only in smokers (Slebos, in press). As Kashmir is not an industrialized region the exposure of the chemicals is bleak particularly aromatic (aryl)-amines such as naphthalene, benzidine, aniline dyes, and

4-aminobiphenyl – known bladder carcinogens (Clavel et al., 2007). With moderate reduction of such workplace exposure, active smoking seems now the strongest environmental risk contributing to most of the bladder cancer cases in Kashmiri population in this study.

The other related risk factor of bladder cancer is age. Our data showed nearly 80% 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 (Jung et al 2007). Ras mutations do not seem to be associated with stage or grade (Theodorescu et al 1991). All the five mutations found were seen randomly in all stages and grades. Thus we could not detect any significant correlation of N-ras mutation with different grades and stages of bladder cancer either between superficial or invasive cancer which is in clear agreement with published data.

The findings presented support the hypothesis that there are distinct molecular pathways that are involved in the pathogenesis of urothelial cell carcinoma and one to a lesser extent involves N-ras gene mutation among other genetic events

Conclusion; To conclude N-ras gene mutations were found in a low frequency in bladder cancer tumors from Kashmir valley, which suggests that N-ras gene is involved in a sub-set of bladder tumors. Nevertheless, these observations a first of its kind from Kashmir need further investigations in a bigger cross section of the bladder cancer patients and relevant controls.

Acknowledgement

The authors gratefully acknowledge the financial support provided by Sher-I-Kashmir Institute of Medical Sciences, Kashmir, for this work We would like to express our gratitude to Ms.Adfar and Ms. Alina of Department of Biochemistry for their technical support and providing useful tips in the laboratory work of this study. Our thanks are also due to the Head and Technical Staff of the operation theater of Department of General Surgery who helped us in the tissue procurement. We also thank the anonymous pathologists of Department of Pathology for the histopathological assessment of the tumor tissues.

References

Barbacid M (1987). Ras genes. *Annu Rev Biochem*, **56**, 779-827.

Bos J (1989). Ras oncogenes in human cancer: a review. *Cancer Res*, **49**, 4682-9.

Bosari S, Marchetti A, Buttitta F, et al (1995) . Detection of p53 mutations by single strand conformation polymorphism (SSCP) gel electrophoresis. A comparative study of radioactive and non radioactive silver-stained SSCP analysis. *Diagn Mol Pathol*, **4**, 249-55.

Clavel J (2007). Progress in the epidemiological understanding of gene environment interactions in major diseases: cancer. *C R Biol*, **330**, 306-17.

Dhar G M, Shah G N, Naheed B, et al (1993). Epidemiological trend in the distribution of cancer in Kashmir Valley. *J Epidemiol Comm Hlth*, **47**, 290-2.

Dinney CP, McConkey DJ, Millikan RE (2004). Focus on

bladder cancer. *Cancer Cell*, **6**, 111-6.

Heney NM (1992). Natural history of superficial bladder cancer. Prognostic features and long-term disease course. *Urol Clin North Am*, **19**, 429-33.

Jemal A, Siegel R, Ward E, et al (2007). Cancer statistics 2007. *CA Cancer J Clin*, **57**, 43-66.

Jemal A, Murray T, Ward E, et al (2005). Cancer statistics 2005. *CA Cancer J Clin*, **55**, 10-30.

Kamarana, NM, Kamat MR, Kurkure AP (2003). National Cancer registry project. ICMR; 2000.

Malumbres M, Barbacid M (2003). Title?? *Nat Rev Cancer*, **3**, 459-65.

Pelucchi C, Bossetti C, Negri E, Malvezzi M, La Vecchia C (2006). The epidemiology of bladder cancer. *Nat Clin Pract Urol*, **3**, 327-40.

Shimizu K, Goldfarb M, Perucho M. & Wigler M(1983). Title?? *Proc Natl Acad Sci USA*, **80**, 383-7.

Zeegeers MP, Kellen E, Buntinx F, van den Brandt PA (2004). The association between smoking, beverage consumption, diet and bladder cancer: a systematic literature review. *World J Urol*, **21**, 392-401.

Joaõ B, Oliveira N, Julie E (2007). NRAS mutation causes a human autoimmune lymphoproliferative syndrome. *Proc Natl Acad Sci USA*, **104**, 8953-8.

Mitin N, Rossman K, Der C (2005). Signaling interplay in Ras super family function. *Curr Biol*, **15**, R563-74

Downward J (2003). Targeting RAS signalling pathways in cancer therapy. *Nature Rev Cancer*, **3**, 11-22 .

Levesque P, Ramchurren N, Saini K(1993) . Screening of human bladder tumors and urine sediments for the presence of H-ras mutations. *Int J Cancer*, **55**, 785-90.

Sameer AS, Chowdhri NA, Abdullah S, Shah ZA, Siddiqi MA(2009). Mutation pattern of K-ras gene in colorectal cancer patients of Kashmir: A report. *Indian J Cancer*, **3**, 219-25.

Trahey M, McCormick F(1987). A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants. *Science*, **238**, 542-5.

Tada M, Omata M, Ohto M (1990). Analysis of ras gene mutations in human hepatic malignant tumors by polymerase chain reaction and direct sequencing. *Cancer Res*, **50**, 1121-4.

Jebar AH, Hurst CD, Tomlinson DC(2005) . FGFR3 and Ras gene mutations are mutually exclusive genetic events in urothelial cell carcinoma. *Oncogene*, **24**, 5218-25.

Sidransky D, Von Eschenbach, A, Tsai YC,(1991) . Identification of p53 gene mutations in bladder cancers and urine samples. *Science*, **252**, 706-9.

Hartge P, Silverman D, Hoover R,(1987) . Changing cigarette habits and bladder cancer risk; a case-control study. *J Natl Cancer Inst*, **78**, 1119-25.

Habuchi T, Takahashi R, Yamada H(1993). Influence of cigarette smoking and schistosomiasis on p53 gene mutation in urothelial cancer. *Cancer Res*, **53**, 3795-9.

Slebos RJ, Hruban RH, Dalesio O, Mooi WJ, Offerhaus GJ (2010). K-ras oncogene activation and smoking in adenocarcinoma of the human lung. *J Natl Cancer Inst*, (in press).

Clavel J (2007). Progress in the epidemiological understanding of gene environment interactions in major diseases: cancer. *C R Biol*, **330**, 306-17.

Jung I, Messing E (2000). Molecular mechanisms and pathways in bladder cancer development and progression. *Cancer Control*, **7**, 325-34.

Theodorescu D, Cornil I, Sheehan C, Man MS, Kerbel RS (1991). Ha-ras induction of the invasive phenotype results in up-regulation of epidermal growth factor receptors and altered responsiveness to epidermal growth factor in human papillary transitional cell carcinoma cells. *Cancer Res*, **51**, 4486-91.