

Ras Oncogene Mutations in Urine Sediments of Patients with Bladder Cancer

Nur Buyru, Hatice Tigli, Faruk Ozcan[†] and Nejat Dalay^{†,*}

Molecular Oncology and Hematopathology Research Center, Cerrahpasa Medical Faculty,

[†]Department of Urology, Istanbul Medical Faculty, and [‡]Oncology Institute, Istanbul University, Istanbul, Turkey

Received 13 January 2003, Accepted 1 March 2003

Early detection of bladder cancer is particularly important since it dramatically affects the survival rates. However, neither urinary cytology nor tumor markers that are currently used are sensitive enough for the early detection of bladder cancer or recurrent disease. The *ras* genes are frequently mutated in cancer. In this study, we investigated the diagnostic potential of *ras* mutation analysis in urinary sediments of patients with bladder cancer using a single-strand conformation polymorphism analysis and polymerase chain reaction. Mutation in codon 12 of the *H-ras* gene was observed in 39% of the patients. Our results indicate that this approach may significantly improve diagnostic sensitivity in detecting bladder tumors.

Keywords: *Ras* mutations, Bladder cancer, Urinary sediments

Introduction

The most frequently detected alterations in oncogenes in both animal and tumor model systems of human cancers are mutations in the *ras* family of oncogenes (Brown *et al.*, 1990; Hoffmann *et al.*, 1993; Krontridis *et al.*, 1993). The *ras* gene family codes for proteins of 21 kD (p21) which are found in the cytoplasm, that is associated with the inner surface of the plasma membrane (Marshall, 1988; Tong *et al.*, 1989). The normal function of the p21 proteins is to interact with tyrosine kinase receptors to activate a signal transduction pathway (So *et al.*, 2001). Therefore, all of the *ras* gene products have GTPase activity, and regulate cell growth and differentiation (Barbacid, 1987). Mutations in members of the *ras* gene family are found in a wide variety of human cancers (Marshall, 1988). Most of these mutations are point mutations

in codon 12, 13, or 61, which convert the *ras* gene to a transforming oncogene (Bos, 1989). The mutated *ras* p21 has a structure that hinders its ability to bind to the GTPase activating protein (GAP), thus keeping p21 in the GTP-bound activated state (Tong *et al.*, 1989; Krenzel *et al.*, 1990).

Patients with bladder cancer, whose disease is detected in an early localized stage, have a 90% chance of surviving at least 5 years (Hruban *et al.*, 1994). If the disease is detected after distant spread, then the 5-year survival rate drops to 9%. Therefore, early detection of bladder carcinoma is extremely important and may have a major impact on the outcome. Unfortunately, the sensitivity of tumor markers for bladder cancer is disappointingly low (Boman *et al.*, 2002).

Urinary cytology has been used as a non-invasive screening method for the detection of urinary tract cancer in the preclinical stage, but it has a number of limitations. For instance, it is much less sensitive than a biopsy, false negative results are obtained in low grade tumors when the specimens are scanty or the tumor cells are masked by larger numbers of nonneoplastic cells (Murphy *et al.*, 1984; Catalona, 1992). Moreover, the interpretation depends on the expertise of the cytopathologist.

Many of the drawbacks of urinary cytologic examination can be overcome by molecular techniques. It has been reported that *H-ras* mutations are frequently observed in bladder cancer (Fujita *et al.*, 1985; Visvanathan *et al.*, 1988; Czerniak *et al.*, 1990; Levesque *et al.*, 1993). Mutation screening in the *ras* genes may be a useful marker for the early detection of bladder cancer. However, to search for the presence of mutations, tumor material should be available. But, fresh tumor samples are usually difficult to obtain in a clinical setting. Therefore, a rapid, sensitive, and non-invasive method may be useful in the diagnosis of bladder cancer, where the low proportion of cancer cells that are present may make their detection difficult. Development of polymerase chain reaction (PCR)-based techniques of lower detection limits has opened the possibility of analyzing samples that contain a low proportion of tumor cells that are admixed with

*To whom correspondence should be addressed.

Fax: 90-216-338-7809

E-mail: ndalay@yahoo.com

a large number of normal cells. An analysis of the urine sediments is practical, non-invasive, and easy to perform. Since DNA can be isolated from the urine sediments by this approach, mutation analysis is more convenient in urological malignancies.

The aim of this study was to investigate the diagnostic utility of the detection of *H-ras* codon 12 mutations in urine samples from the patients with bladder cancer, and to evaluate its potential as a diagnostic tool.

Materials and Methods

In order to identify codon 12 mutations of the *H-ras* gene, DNA was isolated from urine sediments of 33 patients with bladder cancer (mean age 61.7 ± 11.3) and analyzed using PCR and single-strand conformation polymorphism (SSCP). Urine samples from 15 healthy subjects (mean age 40.9 ± 7.3) were used as the control group. The sediments were incubated overnight in a lysis buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, 1% SDS and 100 µg/ml Proteinase K) at 37°C, followed by phenol/chloroform extraction and ethanol precipitation. A 63 bp sequence that spanned codon 12 of the *H-ras* gene was amplified by PCR using primers:

Ras1-5'-GACGGAATATAAGGCTTGTTG-3'

Ras2-5'-TGGATGGTCAGCGCACTCTT-3

The PCR reaction was carried out in a total volume of 25 µl that contained 0.5-1 µg of genomic DNA, 50 mM KCl, 2.5 mM MgCl₂, 10 pmol of each primer, 1 U Taq polymerase (Promega, Madison, USA), 200 µM dNTP mix, and 2 mM Tris-HCl, pH 8.3. The reaction mixture was heated to 94°C for 5 min for the initial denaturation, followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s. The final extension was allowed to proceed for 5 min at 72°C.

For the SSCP analysis, the PCR products were diluted 3-5 times with 95% formamide that contained 10 mM NaOH, 0.05% xylene cyanol loading buffer. Fifteen µl of the diluted sample was denatured at 95°C, quickly chilled on ice, and applied to a 6% non-denaturing polyacrylamide gel that contained 5% glycerol. Electrophoresis was performed at room temperature for 5 h at 170 V. The gels were visualized by silver staining.

To confirm the presence of a single nucleotide substitution at codon 12, which disrupts a restriction site for *Msp* I, the PCR products were digested with *Msp* I and analyzed electrophoretically. Digestion was performed in a total volume of 25 µl that contained 10 µl PCR product, 25 µl 10x digestion buffer, 5 µg BSA, and 10 U *Msp* I (Promega, Madison, USA) by an overnight incubation at 37°C. After digestion, 1 µl of DNA was resolved on 12% non-denaturing polyacrylamide gels in 1x TBE at 100 V for 2 h. Following electrophoresis, the gel was stained in ethidiumbromide and photographed under ultraviolet illumination.

Results

The *ras* gene mutation in the samples was analyzed by amplifying a region that contained codon 12 of the *ras* gene,

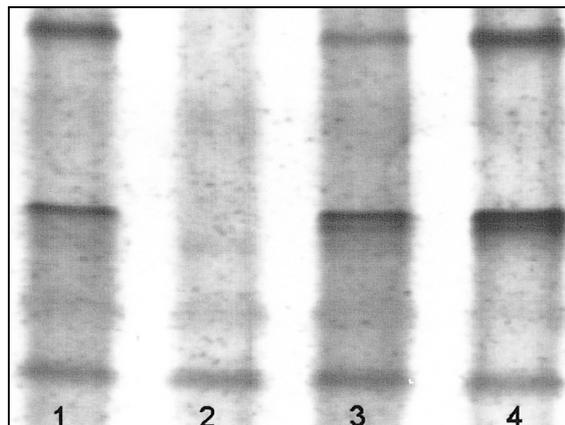


Fig. 1. SSCP analysis of p21 codon 12 in bladder cancer patients. Lanes 1, 3, 4; Normal p21 protein. Lane 2; Mutant sample.



Fig. 2. Restriction enzyme analysis of the mutant samples. Lane 2; Wild-type fragments. Lanes 1, 3, 4, 5; Mutation carrying samples.

and analyzing the PCR fragments by single-strand conformation polymorphism. A SSCP analysis of the PCR products exploits the differential mobility of the DNA fragments that differ by a single-base change in the polyacrylamide gel electrophoresis.

Abnormally migrating bands were observed in 13 (39.4%) samples (Fig. 1). Common bands in Fig. 1 represent different configurations of the reaction product. Since any mutation at the first or second base of codon 12 destroys its restriction site, presence of a codon 12 mutation was confirmed by digestion with the restriction enzyme *Msp* I. While the wild-type gene samples revealed two restriction fragments of 38 and 28 base pairs, the mutation carrying fragments of the *ras* gene remained undigested. All of the specimens that displayed abnormal electrophoretic behaviour harbored a codon 12 mutation. In some patients, the analysis resulted in the detection of both the normal and mutated fragments, but, in most instances, the mutated fragment was selectively

amplified. Representative examples of the specimens that carried the mutation are shown in Fig. 2. In our study group, we observed no association with the stage of the disease.

No mutation was detected in the healthy control group, indicating that the molecular analysis had no false positives.

Discussion

Ras gene mutations have been identified in a variety of tumors, and are able to contribute to the transformation and neoplastic progression of urothelial cells (Knowles and Williamson, 1993). The overexpression of normal and mutated *ras* genes in bladder tumor cell lines induces tumor invasion (Theoderescu *et al.*, 1990). It is estimated that a significant proportion of the bladder tumors have a mutated *ras* gene (Czerniak *et al.*, 1990; Knowles and Williamson, 1993; Burchill *et al.*, 1994). Interestingly, all of the activated *ras* genes in human urothelial tumors have been *H-ras* genes (Visvanathan *et al.*, 1988). Furthermore, the most common *ras* activation event that is associated with bladder tumors is a mutation at codon 12 (Fujita *et al.*, 1985; Visvanathan *et al.*, 1988; Czerniak *et al.*, 1990 and 1992; Levesque *et al.*, 1993; Burchill *et al.*, 1994).

Currently, up to four cystoscopies per year are needed to monitor superficial bladder cancer (van Rhijn *et al.*, 2001). Early detection and follow-up are critical for successful treatment, since a considerable proportion of the cases progress and recur. A diagnosis of bladder cancer can be made by a cytologic examination of urine, but the sensitivity of this technique is very low (Seripa *et al.*, 2001).

Although new tests for tumor antigens in blood or urine have been developed, their accuracy and sensitivity is far from ideal (Sarosdy *et al.*, 1997; Serretta *et al.*, 1998; Wiener *et al.*, 1998; Boman *et al.*, 2002). Urine usually contains cells with oncogene mutations that are characteristic of the related tumor types. Cancer cells in urine are usually mixed with large numbers of genetically-normal epithelial and white blood cells. Since only a small fraction of the cells may contain the mutation, then detection of the *ras* mutation requires a sensitive assay.

Because of their remarkable sensitivity, PCR-based techniques are suitable to detect exfoliated neoplastic cells in the urine of patients with bladder cancer. A SSCP analysis provides a rapid and simple way to screen specific DNA regions, and it is capable of identifying a wide range of mutations. Since clonal *ras* mutations are highly specific for the diagnosis of cancer (Mills *et al.*, 1995), they are beneficial as diagnostic tools in the cases that are positive. Different studies have suggested the clinical utility of the *ras* gene as a biomarker for cancer (Mills *et al.*, 1995; Puig *et al.*, 2000). The resolution of PCR amplification and SSCP can usually detect a mutant product when at least 10-20% of the cells carry the mutation (Levi *et al.*, 1991).

Our findings suggest that this technique offers a useful

alternative for a non-invasive diagnosis in a significant number of patients. Selective amplification of the mutation-carrying fragment, presumably by suppressing its normal counterpart and by factors influencing translation rates, facilitates the detection of tumor cells. The frequency of the codon 12 mutation was 39% in the present study. This is higher than some studies (Levesque *et al.*, 1993; Saito *et al.*, 1997; Olderoy *et al.*, 1998), but agrees with several reports on the bladder (Czerniak *et al.*, 1990; Fitzgerald *et al.*, 1995), colon (Kopreski *et al.*, 1997; Puig *et al.*, 2000), and lung (Mills *et al.*, 1995) tumors. Considerably higher frequencies have also been reported (Burchill *et al.*, 1991; Burchill *et al.*, 1994; Przybojewska *et al.*, 2000).

Since our assay detected mutations in 39% of the patients, this test could be useful in a considerable number of patients with bladder cancer by providing a clinically valuable cancer marker and improving the lower diagnostic yield of cystoscopy. The higher sensitivity of the test may allow the detection of cancer even before cytological evidence. Studies that would be comprised of larger groups of patients are warranted in order to investigate the association of the detection rate with the stage of the disease.

Altogether, our findings indicate that the detection of *ras* mutations in voided urine (as an adjunct to a cytologic examination) may substantially improve the sensitivity of detecting bladder tumors. It is particularly important that this non-invasive technique offers a higher sensitivity and specificity without adding false positives. Our study demonstrates that the potential use of this molecular analysis could be especially useful in patients to whom the cystoscopic examination is inconclusive or cannot be adequately performed. A mutation analysis in urine samples might be useful in improving the detection of existing cancer and for monitoring patients with recurrent disease.

Acknowledgments This study was supported by the Istanbul University Research Fund, Project Nr: B-1063.

References

- Barbacid, M. (1987) *ras* genes. *Annu. Rev. Biochem.* **56**, 779-827.
- Boman, H., Hedelin, H. and Holmang, S. (2002) Four bladder tumor markers have a disappointingly low sensitivity for small size and low grade recurrence. *J. Urol.* **167**, 80-83.
- Bos, J. L. (1989) *Ras* oncogenes in human cancer: a review. *Cancer Res.* **48**, 4682-4689.
- Brown, K., Buchmann, A. and Balmain A. (1990) Carcinogen-induced mutations in the mouse *c-Ha-ras* gene provide evidence of multiple pathways for tumor progression. *Proc. Natl. Acad. Sci. USA* **87**, 538-542.
- Burchill, S. A., Lunec, J., Mellon, K. and Neal, D. E. (1991) Analysis of *H-ras* mutations in primary human bladder tumors. *Br. J. Cancer* **63**, 62.
- Burchill, S. A., Neal, D. E. and Lunec, J. (1994) Frequency of *H-ras* mutations in human bladder cancer detected by direct

- sequencing. *Br. J. Urol.* **73**, 516-521.
- Czerniak, B., Cohen, G. L., Etkind, P., Deitch, D., Simmons, H., Herz, F. and Koss, L. G. (1992) Concurrent mutations of coding and regulatory sequences of the *H-ras* gene in urinary bladder carcinomas. *Hum. Pathol.* **23**, 1199-1204.
- Czerniak, B., Deitch, D., Simmons, H., Etkind, P., Herz, F. and Koss, L. G. (1990) *Ha-ras* gene codon 12 mutation and DNA ploidy in urinary bladder carcinoma. *Br. J. Cancer* **62**, 762-763.
- Fitzgerald, J. M., Ramchurren, N., Rieger, K., Levesque, P., Silverman, M., Libertino, J. A. and Summerhayes, I. C. (1995) Identification of *H-ras* mutations in urine sediments complements cytology in the detection of bladder tumors. *J. Natl. Cancer Inst.* **87**, 129-133.
- Fujita, J., Srivastava, S. K., Kraus, M. H., Rhim, J. S., Tronick, S. R. and Aaronson, S. A. (1985) Frequency of molecular alterations affecting *ras* protooncogenes in human urinary tract tumors. *Proc. Natl. Acad. Sci. USA* **82**, 3849-3853.
- Hoffmann, J. S., Fry, M., Williams, J. and Loeb, L. A. (1993) Codon 12 and 13 of *H-ras* protooncogene interrupt the progression of DNA synthesis catalyzed by DNA polymerase alpha. *Cancer Res.* **53**, 2895-2900.
- Hruban, R. H., van der Riet, P., Erozan, Y. S. and Sidransky, D. (1994) Brief report: Molecular biology and the early detection of carcinoma of the bladder-The case of Hubert H. Humphrey. *N. Engl. J. Med.* **330**, 1276-1278.
- Knowles, M. A. and Williamson, M. (1993) Mutation of *H-ras* is infrequent in bladder cancer: Confirmation by single-strand conformation polymorphism analysis, designed restriction fragment length polymorphisms and direct sequencing. *Cancer Res.* **43**, 133-139.
- Kopreski, M. S., Benko, F. A., Kwee, C., Leitzel, K. E., Eskander, E., Lipton, A. and Gocke, C. D. (1997) Detection of mutant *K-ras* DNA in plasma or serum of patients with colorectal cancer. *Br. J. Cancer* **76**, 1293-1299.
- Krengel, U., Schlichting, L., Scherer, A., Schumann, R., Frech, M., John, J., Kabsch, W., Pai, E. F. and Wittinghofer, A. (1990) Three-dimensional structures of *H-ras* p21 mutants: Molecular basis for their inability to function as signal switch molecules. *Cell* **62**, 539-548.
- Krontiris, T. G., Devlin, B., Karp, D. D., Robert, N. J. and Rish, N. (1993) An association between the risk of cancer and mutations in the *HRAS1* minisatellite locus. *N. Engl. J. Med.* **329**, 517-523.
- Levesque, P., Ramchurren, N., Saini, K., Joyce, A., Libertino, J. and Summerhayes I. C. (1993) Screening of human bladder tumors and urine sediments for the presence of *H-ras* mutations. *Int. J. Cancer* **55**, 785-790.
- Levi, S., Urbano-Ispizua, A., Gill, R., Thomas, D. M., Gilbertson, J., Foster, C. and Marshall, C. J. (1991) Multiple *K-ras* codon 12 mutations in cholangiocarcinomas demonstrated with a sensitive polymerase chain reaction technique. *Cancer Res.* **51**, 3497-3502.
- Marshall, C. J. (1988) The *ras* oncogenes. *J. Cell Sci.* **10** (suppl), 157-169.
- Mills, N. E., Fishman, C. L., Scholes, J., Anderson, S. E., Rom, W. N. and Jacobson, D. R. (1995) Detection of *ras* oncogene mutations in bronchoalveolar lavage fluid for lung cancer diagnosis. *J. Natl. Cancer Inst.* **87**, 1056-1060.
- Murphy, W. M., Soloway, M. S., Jukkola, A. F., Crabtree, W. N. and Ford, K. S. (1984) Urinary cytology and bladder cancer: the cellular features of transitional cell neoplasms. *Cancer* **53**, 1555-1565.
- Olderoy, G., Daehlin, L. and OGREID, D. (1998) Low frequency mutation of *Ha-ras* and *Ki-ras* oncogenes in transitional cell carcinoma of the bladder. *Anticancer Res.* **18**, 2675-2678.
- Przybojewskai, B., Jagiello, A. and Salmuzna, P. (2000) *H-ras*, *K-ras* and *N-ras* gene activation in human bladder cancers. *Cancer Genet. Cytogenet.* **121**, 73-77.
- Puig, P., Urgell, E., Capella, G., Sancho, F. J., Pujol, J., Boadas, J., Farre, A., Lluís, F., Gonzalez-Sastre, F. and Mora, J. (2000) A highly sensitive method for *K-ras* mutation detection is useful in diagnosis of gastrointestinal cancer. *Int. J. Cancer* **85**, 73-77.
- van Rhijn, B. W. G., Lurkin, I., Kirkels, W. J., van der Kwast, T. H. and Zwarthoff, E. C. (2001) Microsatellite analysis- DNA test in urine competes with cystoscopy in follow-up of superficial bladder cancer. *Cancer* **92**, 768-775.
- Saito, S., Hata, M., Fukuyama, R., Sakai, K., Kudoh, J., Tazaki, H. and Shimizu, N. (1997) Screening of *H-ras* gene point mutations in 50 cases of bladder carcinoma. *Int. J. Urol.* **4**, 178-185.
- Sarosdy, M. F., Hudson, M. A., Ellis, W. J., Soloway, M. S., deVere W. R., Sheinfeld, J., Jarowenko, M. V., Schellhammer, P. F., Schervish, E. W., Patel, J. V., Chodak, G. W., Lamm, D. L., Johnson, R. D., Henderson, M., Adams, G., Blumenstein, B. A., Thoeke, K. R., Pfalzgraf, R. D., Murchison, H. A. and Brunelle, S. L. (1997) Improved detection of recurrent bladder cancer using the Bard BTastat test. *Urology* **50**, 349-353.
- Seripa, D., Parrella, P., Gallucci, M., Gravina, C., Papa, S., Fortunato, P., Alcini, A., Flammia, G., Lazzari, M. and Fazio, V. M. (2001) Sensitive detection of transitional cell carcinoma of the bladder by microsatellite analysis of cells exfoliated in urine. *Int. J. Cancer* **95**, 364-369.
- Serretta, V., Lo Presti, D., Vasile, P., Gange, E., Esposito, E. and Menozzi, I. (1998) Urinary NMP22 for the detection of recurrence after transurethral resection of transitional cell carcinoma of the bladder. *Urology* **52**, 793-796.
- So, E. Y., Jang, J. Y. and Lee, C. E. (2001) Cross-talk between STAT6 and ras/MAPK pathway for the IL-4-mediated T cell survival. *J. Biochem. Mol. Biol.* **34**, 578-583.
- Theoderescu, D., Cornil, I., Fernandez, B. J. and Kerbel, R. S. (1990) Overexpression of normal and mutated forms of *H-ras* induces orthotopic bladder invasion in a human transitional cell carcinoma. *Proc. Natl. Acad. Sci. USA* **87**, 9047-9051.
- Tong, L., Milburn, M. V., deVos, A. M. and Kim, S. H. (1989) Structure of *ras* protein. *Science* **245**, 244.
- Tong, L., de Vos A. M., Milburn, M. V., Jancarik, J., Noguchi, S., Nishimura, S., Miura, K., Ohtsuka, E. And Kim, S. H. (1989) Structural differences between a *ras* oncogene protein and the normal protein. *Nature* **337**, 90-93.
- Visvanathan, K. V., Pocock, R. D. and Summerhayes, I. C. (1988) Preferential and novel activation of *H-ras* in human bladder carcinomas. *Oncogene Res.* **3**, 77-86.
- Wiener, H. G., Mian, C., Jaitel, A., Pycha, A., Schatzl, G. and Marberger, M. (1998) Can urine bound diagnostic tests replace cystoscopy in the management of bladder cancer? *J. Urol.* **159**, 1876-1880.
- Young, M. J. and Soloway, M. S. (1998) Office evaluation and management of bladder neoplasms. *Urol. Clin. North Am.* **25**, 603-611.