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

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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
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 \( H\text{-ras} \) 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 \( H\text{-ras} \) 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 \( H\text{-ras} \) gene was amplified by PCR using primers:

- Ras1-5' GACGGAA TA TAAGGCTTGTTG-3'
- Ras2-5' TGGA TGGTCAGCGCACTCTT-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 µM 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.

**Results**

The \( ras \) gene mutation in the samples was analyzed by amplifying a region that contained codon 12 of the \( ras \) gene, 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 behavior 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

**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.
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; Oldroy 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; Przybojeswska 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.

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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. 49, 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.
Czer尼亚k, 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.
Czerniaik, B., Deitch, D., Simmons, H., Etkind, P., Herz, F. and Koss, L. G. (1990) H-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. (1990) Three-dimensional structures of H-ras/MAPK pathway for the IL-4-mediated T cell survival. Proc. Natl. Acad. Sci. USA 87, 9047-9051.
Konn, S., Kusumoto, K., Kanai, T., Sugano, M., Muto, Y., Takai, S., Takada, Y., Kamimura, K. and Kurobe, A. (1993) Improved detection of recurrent bladder cancer using the BarD BTAXtest. Urol. 50, 349-353.
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 HRAS 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., Urbanio-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 Ogred, D. (1998) Low frequency mutation of Ha-ras and Ki-ras oncogenes in transitional cell carcinoma of the bladder. Anticancer Res. 18, 2675-2678.
Przybojewski, 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., Lluis, 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., Kirikels, 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., Tholke, K. R., Pfalzgraf, R. D., Murchison, H. A. and Brunelle, S. L. (1997) Improved detection of recurrent bladder cancer using the Bard BTAXtest. Urol. 50, 349-353.
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., Tholke, K. R., Pfalzgraf, R. D., Murchison, H. A. and Brunelle, S. L. (1997) Improved detection of recurrent bladder cancer using the Bard BTAXtest. Urol. 50, 349-353.
Seripa, D., Parrella, P., Gallucci, M., Gravina, C., Papa, S., Fortunato, P., Alcini, A., Flammini, 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., deVos A. M., Milburn, M. V., Jancaik, J., Naguchi, 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.
Viswanathan, 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.