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A PCR-RFLP Method for the Detection of Activated H-ras Oncogene with a Point Mutation at Codon 12 and 61

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To investigate the incidence of the H-ras gene activation in bladder tumor and the feasibility of using urinary washout samples for screening, a series of 33 human bladder tumors and their preoperatively collected urinary washout samples were screened using a mutant specific PCR-RFLP (polymerase chain-restriction fragment length polymorphism) to detect a point mutation of the H-ras gene. Five tumors were found to harbor H-ras mutations where two tumors had a glycine to valine (G→T) change in codon 12 and three tumors had a glutamine to lysine (C→A) change in codon 61, respectively. Moreover, we could also detect the same point mutations of the H-ras gene in corresponding urine washout samples. The incidence of H-ras mutation in Korean bladder cancer was estimated at approximately 15.2%.

In conclusion, a mutant specific PCR-RFLP method for the detection of H-ras gene mutation is useful for screening or postoperative follow-up of bladder tumor due to its simplicity and high specificity even in urinary samples.

Key Words: H-ras gene, bladder tumor, point mutation, PCR-RFLP

The ras gene family is composed of three closely related genes (H-ras, K-ras, and N-ras), which have been the most widely studied in human tumors (Barbacid, 1987; Bos, 1989; Barbacid, 1990). Among them, the H-ras gene was first found playing an important role in the etiology of a significant portion of human cancers (Fujita *et al.* 1988; VisVanathan *et al.* 1988). Early studies using the NIH/3T3 transfection assay to assess the frequency of ras activation in human urothelial tumors revealed H-ras activation rates of 6~17% (Fujita *et al.* 1985; VisVanathan, 1988). From these studies it was concluded that the H-ras gene was activated in urothelial cell carcinoma as characterized by a point mutation at codon 12, 13 and 61 in approximately 20% of bladder tumors (Sigal *et al.* 1986; Walter *et al.* 1986). However a more recent study, using the polymerase chain reaction and oligonucleotide probing, reported the detection of H-ras activation events in 12 of 33 (36%) bladder tumors where the only mutation found was a glycine-to-valine change at codon 12 (Czerniak *et al.* 1990). Consistent with this observation, Burchill *et al.* (1991) reported that 38 of 50 bladder tumors (76%) were positive for mutations in H-ras gene with 27 tumors (54%) harboring the glycine-to-valine change at codon

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12. In the most extensive study to date involving 152 bladder tumors, Knowles and Williamson (1993) reported H-ras activation in only 9 tumors (6%) in which glycine-to-valine change at codon 12 was observed. But it is clear that the prevalence of H-ras activation in bladder carcinoma is a contentious issue.

As mentioned above conflicting data have been reported regarding the incidence and significance of ras oncogene mutations in bladder cancer. Recent application of polymerase chain reaction (PCR) technology simplified the analysis of mutations and increased the sensitivity for the detection of specific mutations even in a small number of cells (Bos, 1989; Yasuhiki *et al.* 1990). Recently polymerase chain reaction and hybridization assay with allele-specific oligonucleotide (ASO) probes allowed precise analysis of point mutation in a wide variety of human cancer tissues and cell lines (Enomoto *et al.* 1990; Grimmond *et al.* 1992). A simple method to detect the point mutations at codon 12 or 61 of H-ras genes was developed (Bos, 1989; Yasuhiki *et al.* 1990). This is a PCR-RFLP method with enhanced sensitivity for the detection of mutant H-ras alleles. The high sensitivity was achieved by selective PCR of mutant H-ras gene sequences using a two-stage procedure. This method is useful to detect the point mutations in cells released in urine or paraffin sections of the bladder tumor tissues.

In this study, we assessed the incidence of H-ras mutation in tumor and urinary sediment from 33 patients with bladder cancer using highly sensitive mutant specific double PCR-RFLP in Korea. The mutation sequence was then determined by direct sequencing.

MATERIALS AND METHODS

Materials

Bladder tumor tissues were obtained from 33 patients undergoing transurethral resection and bladder wash out at our department. The specimens were immediately snap-frozen in liquid nitrogen and then stored at −70℃. Pathologic specimens were reviewed by a specialized pathologist. To test the reliability and sensitivity of our newly designed method, we used the DNA extracted from T24 bladder cancer cell line with the point mutation at codon 12 and SK2 malignant melanoma cell line with the point mutation at codon 61 as the positive control cell lines. Codon 12 of T24 cells were mutated from GGC (Gly) to GTC (Val), codon 61 of SK2 cells were mutated from CAG (Gln) to CTG (Leu).

DNA preparation

Bladder tumor tissues were washed in PBS (phosphate-buffered saline), snap-frozen in liquid-nitrogen, and stored at −70℃. Frozen tissues were thawed, quickly minced, and lysed in a buffer solution containing 100 mM NaCl, 10 mM Tris-HCl(pH 8.0), 25 mM EDTA(pH 8.0), 0.5% SDS, and 0.1 mg/ml Proteinase K. Tissues were digested overnight at 37℃. High molecular weight DNA was then isolated by phenol/chloroform extraction and ethanol precipitation.

DNAs were extracted by these standard procedures from 33 fresh tumor samples, urine washout samples, and control cell lines.

Sensitivity test

Detection of the H-ras gene point mutation mixed in substantial amounts of normal DNA was attempted using various samples of DNA mixtures (Fig. 1). DNA mixtures were amplified with PCR and digested by NaeI or Bst NI. The DNA component with the point mutation at codon 12, which was derived from human bladder cancer T24 or SK2 cells, remained uncleaved. While normal DNA component, which was derived from normal adrenal gland tissue, was cleaved into two bands of 95 base pair(bp) and 69 bp.

Mutant specific PCR-RFLP method

Amplifications with Taq polymerase were performed in 30 μl reaction mixtures containing 0.2 unit of Taq polymerase, 3 mM dNTP, and 6 pmole of each primer. The reaction mixtures were subjected to amplification cycle of 95℃ for 1 min, 57℃ for 1.5 min and 72℃ for 2 min.

	2	3	4	5	6	7	8	9
Normal	0					499.5		
T24 or SK2	500					0.5		

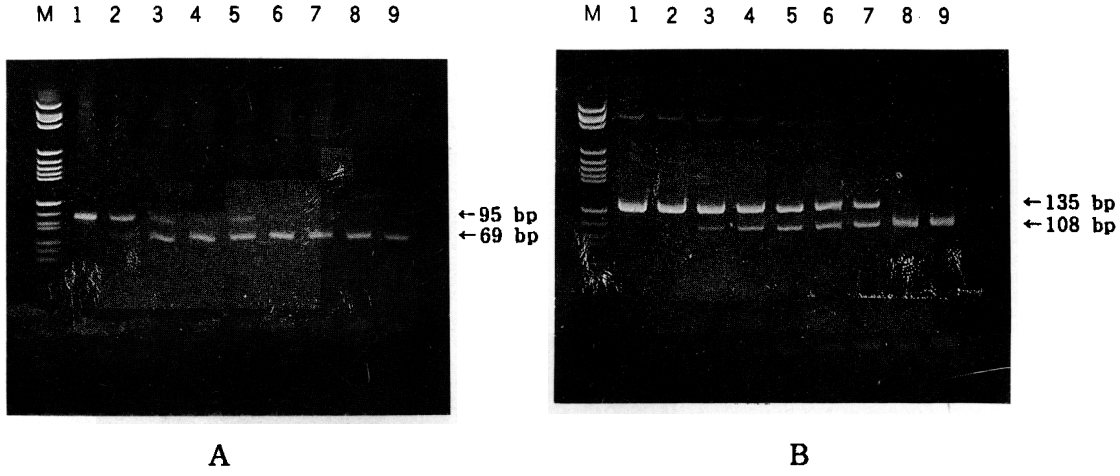

Fig. 1. *Feasibility test of the mutant specific PCR-RFLP analysis. DNA extracted from T24 (A) or SK (B) cell lines were mixed with DNA extracted from normal cell line with ratios as indicated in each lane. T24 and SK2 were used for positive control. T24 is a cell line of human malignant melanoma cell line mutate to CTG (Leu) from GGC (Gly) at codon 12 of H-ras and SK2 is human malignant melanoma cell line mutated to CTG (Leu) from CAG (Gln) at codon 61.*

Table 1. Oligonucleotide primers used in this study

Primer	Sequence(5′→3′)
Outer primer of point mutation at codon 12	
Sense	AGGAGCGATGACGGAATATAAGC
Antisense	GGCTCACCTCTATAGTGGGGTCGTATT
Inner primer of point mutation at codon 12	
Sense	AATATAAGCTGGTGGTGGTGGGCGC
Antisense	GGGGTCGTATTCGTCCACAAAATG
Primer of point mutation at codon 61	
Sense	TGCCTGTTGGACATCCGGGATACCGCC
Antisense	CTGGTGGATGTCCTCAAAAGACTTG

For experiments in which a single PCR was performed (in case of point mutation at codon 61), 30 cycles of amplification was done. For experiments in which two PCR amplifications were performed (in case of point mutation at codon 12), the first PCR comprised 14 cycles, followed by restriction enzyme digestion and then a second PCR of 16 cycles, total 30 cycles; 1 μl of the first digest was used as template for the second PCR.

Restriction enzyme analysis

Nae I cleavage site (GCCGGC) overlaps the nucleotides of codon 12 of the H-ras gene.

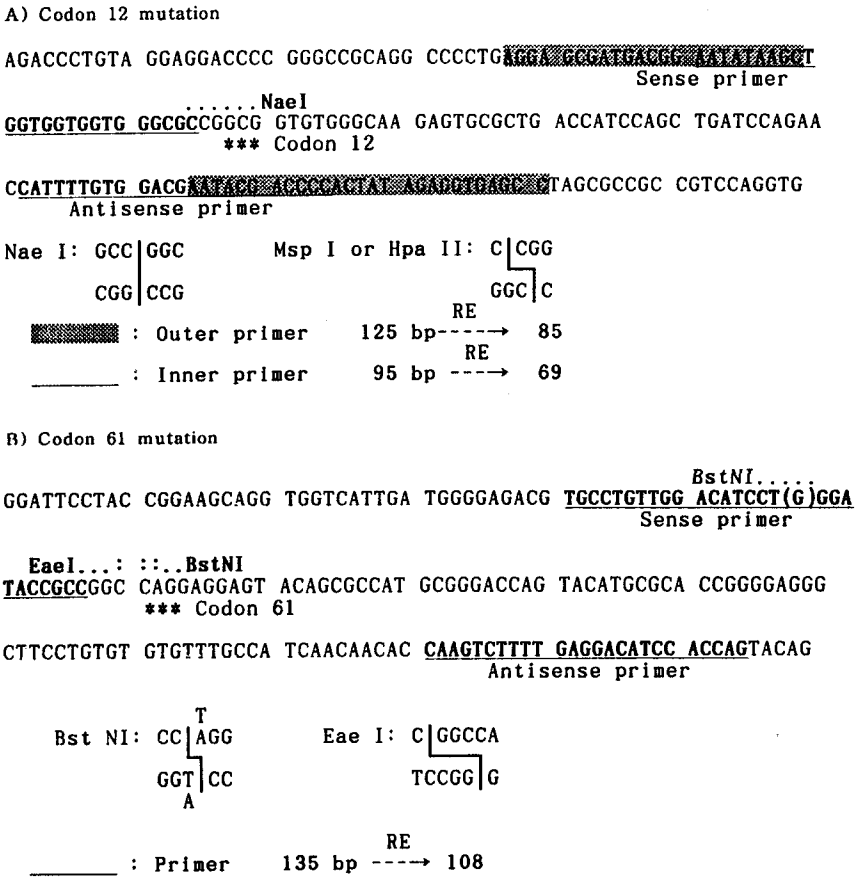

Fig. 2. *The nucleotide sequence of H-ras gene around codon 12 (A) and codon 61 region. Sense primers and antisense primers used in the PCR analysis are indicated, The codon 12 and 61 are indicated by asterisk(*). Relevant restriction enzyme sites and the size of expected PCR product before and after restriction enzyme digestion (RE) are shown above.*

This Nae I cleavage site is lost when a mutation occurs at codon 12. Of the two Bst NI cleavage sites (CCAGG and CCTGG) in the DNA segment one overlaped with the nucleotides of codon 61 and the other was unrelated to codon 61 so we replaced this unrelated codon in the sense primer (CCTGG→CCGGG). Msp I cleavage site (CCGG) and Eae I cleavage site (CGGCCA) was also used at codon 12 and 61, respectively.

The reaction mixture for the digestion with these restriction enzymes contained 8 μl of amplified DNA, 1 unit of restriction enzyme and 2.0 μl of 10 x buffer and 9.0 μl of distilled water in a total volume of 20 μl. This mixture was incubated at 37℃ for 1 hour.

The PCR products were resolved by electrophoresis in 13% polyacrylamide gel with 120V for 45 minutes and were visualized by staining with ethidium bromide.

Direct sequencing

PCR products were electrophoresed on 2 % NuSieve agarose gel and extracted with Qiaex (Qiagen, Chats-worth, CA, USA). PCR products were sequenced according to a modification of the sequenase protocol (US Biochemical, Cleveland, OH, USA).

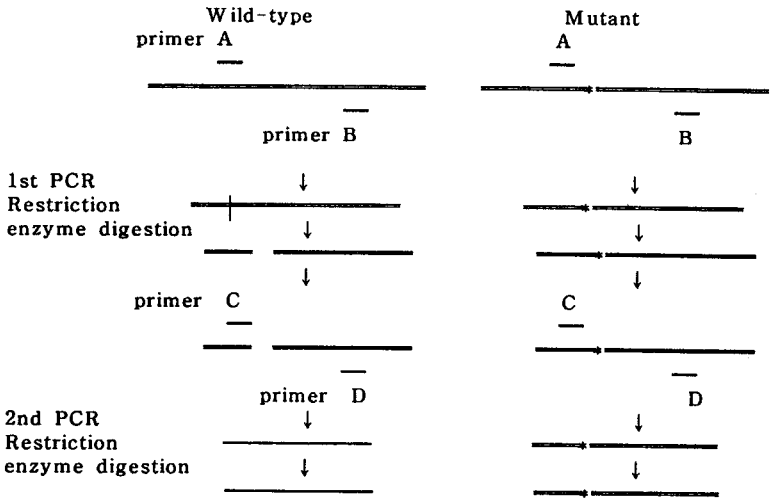

Fig. 3. *The strategy used for the mutant specific PCR-RFLP analysis of H-ras oncogenes. The DNA, which contains a mutation (*), is amplified using outer primers A and B. Incubation with the restriction enzyme cleaves the amplified wild-type sequence and leaves mutant sequence intact. The inner primers C and D are used in the second PCR, and the uncleaved mutant sequence is amplified. The products of the second PCR are incubated with the restriction enzyme. The restriction enzyme-resistant DNA fragment is diagnostic for the presence of a mutant.*

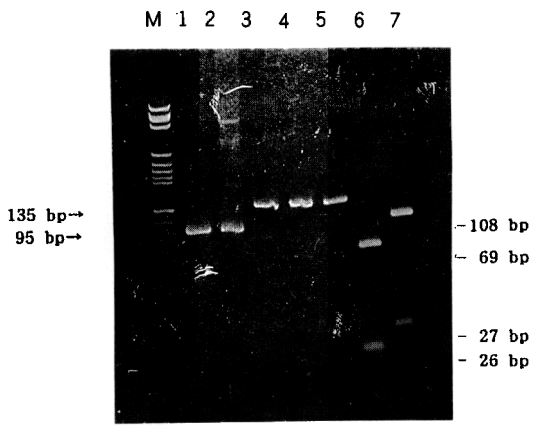

Fig. 4. *Detection of the mutated H-ras gene in tumor samples by PCR-RFLP analysis. M; pBR322/ Hae III marker. Lane 1; RT 59, Lane 2; RT 99, Lane 3; RT 03, Lane4; RT 4, Lane 5; RT 88, Lane 6; normal (codon 12), Lane 7; normal (codon 61).*

RESULTS

Specimens from 33 transitional carcinoma of bladder were examined by the PCR-RFLP methods for detection of the point mutation at codon 12 or 61. Five tumors were found to harbor H-ras mutations where two tumors (6 %) had a glycine to valine (G→T) change at codon 12 and three tumors (9%) had a glutamine to lysine (C→A) change at codon 61, respectively. The mutation sites of tumor specimens were the same as those of urinary sediments in all five patients with mutations respectively (Fig. 4). Two tumors (Lane 1, 2) which had mutations at codon 12 showed a band at 95 bp, one specimen which was normal at codon 12 showed 69 and 26 bp band cleavage. Three tumors (Lane 3, 4, 5) which had mutations at codon 61 showed a band at the 135 bp region, one specimen which was normal at codon 61 showed 108 and 27 bp band regions cleavage(Fig. 5).

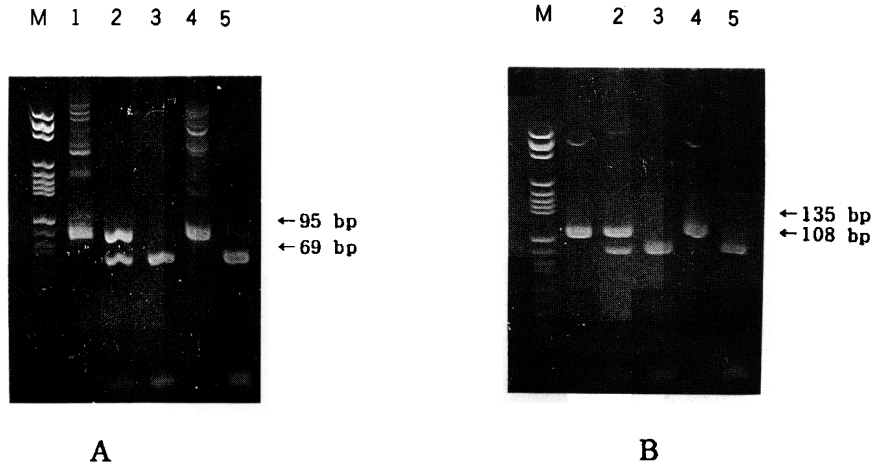

Fig. 5. *Detection of the mutated H-ras gene in tumor and its washout sample. Analysis of PCR band. A; codon 12, B; codon 61, M; pBR322/Hae III marker. Lane 1; tumor sample, Lane 2; wash out sample, Lane 3; normal mucosa sample, Lane 4; positive control (cell line T24 or SK2), Lane 5; negative control (normal DNA).*

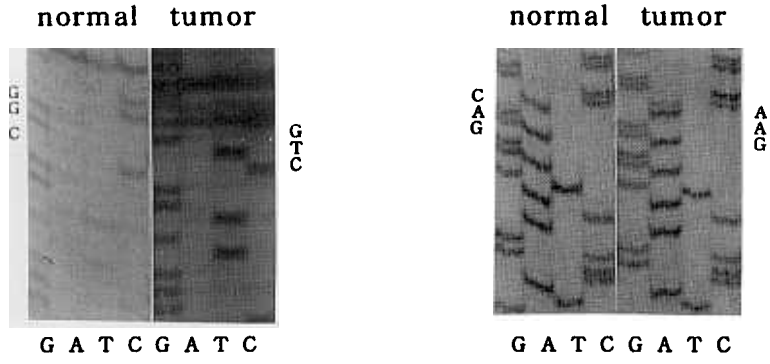

Fig. 6. *Direct sequencing analysis of the PCR products derived from the mutated H-ras gene. A. Two tumors had a glycine to valine (G→T) change in codon 12. B. Three tumors had a glutamine to lysine (C→A) change in codon 61.*

The sequence of five mutated specimens were determined using direct sequencing(Fig. 6).

DISCUSSION

Over the past decade, intense research led to the discovery that cellular genetic alterations are associated with neoplasia and are probably the main cause of cancer (Bishop, 1983; Slamon, 1984). Gene transfer assays showed about 20% of human neoplasms contained oncogenes that would induce malignant transformation of various rodent cells and many of these transforming genes were found to be members of the Ras gene family (Fujita *et al.* 1985; Nobuo *et al.* 1989). The Ras genes, the most widely studied to date, consists of three genes, designated H-Ras, K-Ras, and N-ras. Ras genes are activated from the normal

proto-oncogene sequence by single point mutation. This mutation alter the Ras protein signal transduction pathway leading to unregulated cellular growth (Taparowsky *et al.* 1983; Kraus *et al.* 1984; Yuasa *et al.* 1984). H-ras gene located on the short arm of chromosome 11 is reported to be the most commonly activated in cases of human urinary tract tumors (Heim and Mitelman, 1987). The incidence of point mutation of the H-ras gene in bladder cancer is estimated to be 5~17% using the NIH/3T3 transfection assay (Der *et al.* 1986). This rate is rather low compared with other cancers such as pancreatic cancer and colorectal cancer (Bos *et al.* 1987). From these early studies it was concluded that H-ras was preferentially activated in urothelial-cell carcinomas. And the genetic changes were characterized by point mutational changes at codon hotspots 12, 13, and 61 in less than 20% of bladder tumors.

More recently, because of advances in molecular biology techniques, the incidence of Ras mutations in various animal and human neoplasms have been reported (Bos, 1989; Barbacid, 1990). Mutations at the 12 and 61 codon have been the most frequently seen in human tumors but the incidence of these mutations vary widely depending on the type of neoplasia (Yuasa *et al.* 1984; Barbacid, 1987; Barbacid, 1990). The exact role of the Ras oncogenes in carcinogenesis remains to be determined, although clinical work with colon tumors and recent animal work suggests that these mutations may be an early event and may precede frank neoplasia (Enomoto *et al.* 1990). It is believed however that Ras mutations alone are not sufficient to induce human cancers but that other oncogenes and/or tumor suppressor genes or other promoting agents are involved in a multistep phenomenon (Land *et al.* 1983).

Though we examined all samples with more than 50% of cancer cells on a morphological basis, small groups with mutated genes might have been missed. And because DNA transfection assay can detect more than 1,000-fold differences in transforming efficiency among members of the mutated ras genes (Der *et al.* 1986), it is likely that this method missed the detection of a point mutation. The incidence of ras gene mutations in bladder cancer may be found in different rates if investigated with other methods (Shiro, 1992). So the diagnosis of many germline and somatic disease became possible through the development of techniques for the detection of molecular lesions.

Recent applications of polymerase chain reaction (PCR) technology has simplified analysis of mutations and increased the sensitivity for detecting specific mutations even in a small number of cells (Bos *et al.* 1987; Shiro, 1992). We designed a novel PCR method to detect activated H-ras oncogene with a point mutation at codon 12 or codon 61, respectively. To increase the sensitivity of the PCR analysis for the detection of the activated H-ras gene, the first PCR products were digested with a restriction enzyme that can cleave wild-type but not mutant H-ras gene. By using this method, we were able to accurately detect the point mutation of H-ras gene even in urine specimens. In this study, the detection of point mutations at codon 12 or codon 61 of H-ras gene was attempted by the simple method involving double PCR amplification. Adequate conditions for PCR, restriction enzyme digestion, and dot blot hybridization were established using cloned H-ras gene DNAs as a model system. Then, DNAs isolated from frozen tumor specimens of bladder tumor were used. An H-ras gene point mutation in frozen specimen and urine cells was readily detected by our method. Therefore, the point mutation was detected in urine cells and that 0.1 % of mutated DNA mixed in normal DNA was detectable, suggesting the possibility of molecular diagnosis for the occurrence or recurrence of bladder cancer in an early stage (Bonfanti *et al.* 1988; Enomoto *et al.* 1990).

Epidemiologic studies have indicated that bladder cancer can be associated with exposure of carcinogen (Balmain *et al.* 1984; Barbacid, 1986). It is well known that N-methyl N-nitrosourea (MNU) leads to G→C to A→T base transition at codon 12. The 7,12-dimethylbenzanthracene (DMBA) results in mutations of adenine residues at codon 61 (Barbacid,

1986). Zarbl *et al.* (1985) detected activated H-ras oncogenes in MNU-induced rat mammary carcinoma and DMBA-initiated mouse skin carcinoma. Workers exposed to some organic compounds are thought to have a high risk for the occurrence of bladder cancer, and therefore examining the incidence of a point mutation in H-ras gene of the bladder cancer tissues from these workers will be of great interest. In this report, we also report the incidence of H-ras activation in Korean bladder tumors with adequate numbers of candidates for the first time. The incidence in Koreans is not so high as that of recent reports in other countries showing no significant biologic difference between Koreans and the others. But this requires further studies.

In conclusion, this newly designed method provides a simple, sensitive, and accurate analysis of H-ras gene mutation even in the urine washing cytology. The method should be useful for the early diagnosis of occurrence or recurrence of bladder cancer.

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