H-*ras* oncogene mutations in the urine of patients with bladder tumors: description of a non-invasive method for the detection of neoplasia

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Abstract. Bladder cancers are usually curable, by surgical or transurethral excision, if diagnosed at an early stage. Tumor derived mutations in oncogenes potentially provide specific markers for the detection of surgically resectable tumors. The detection of point mutations of H-*ras* oncogene correlated with this disease. DNA sequences produced by the Polymerase Chain Reaction (PCR) can be considered for this application, because theoretically bladder tumors should shed cells containing this mutation into the urine. We examined urine from 21 individuals with bladder cancer before any treatment, as well as tissue specimens from the excised tumor and we found 10 mutations of the H-*ras* gene at codon 12 in the urine (47.61%) and 14 mutations in the tumor specimens (66.66%). We were able to detect nearly 50% of the patients with bladder tumors using this method. We also studied two relapses; in one case (which presented the mutation in the original tumor and the urine) the relapse grade had progressed from II to III. In the other case the relapse grade stayed at III but it presented for the first time the studied mutation in the urine. These results provide the theoretical and technical basis for the detection of bladder tumors by a non-invasive method and possibly for the evaluation of the invasiveness of the disease.

Introduction

Bladder cancer is a common malignancy in the world with 180,000 new cases expected in 1992. Whereas individuals with advanced disease have a poor prognosis, bladder tumors diagnosed in an earlier stage can usually be cured with transurethral or surgical excision. Only 10 to 15% of patients with superficial bladder cancer subsequently develop invasive or metastatic disease (1,2), however, approximately 70% of patients have one or more tumor recurrences (3), which express a higher histologic grade in about 25% of cases. Methods to detect surgically resectable tumors could therefore reduce deaths from the disease. Tumor derived mutations in oncogenes potentially provide specific markers for such a detection.

Oncogenes are highly conserved genes in any living organism and have important functions in cellular proliferation and differentiation. Loss of regulation in this complex system, resulting from structural modification of a normal gene or of its controlling regions, is called oncogene activation. A family of such genes that is frequently found to harbour a mutation in human tumors is that of *ras* genes (4). This family consists of three functional genes: H-*ras*, K-*ras* and N-*ras*, which encode highly similar proteins with molecular weights of 21,000. Mutated *ras* genes were first identified by their ability to transform NIH/3T3 cells after DNA transfection. Subsequent analysis of a variety of tumor samples revealed that some human tumors had one of the three *ras* genes harbouring a point mutation; as a result, the protein produced has an altered amino acid residue at one of the critical positions 12, 13 or 61.

H-*ras* oncogene activations are known to occur during the process of bladder carcinogenesis in humans, along with the tumor progression. *In vitro* experiments have shown that the transfection of the H-*ras* oncogene into a low metastatic epithelial cell line results in the acquirement of significantly increased metastatic capacity (5) and also that the malignant properties of cellular sublines selected from a human bladder cancer cell line which contains an activated H-*ras* oncogene are highly enhanced (6). Other experiments have proved the activation of this oncogene in rat bladder tumors induced by the N-butyl-N-(4-hydroxybutyl)nitrosamine (7). However, no specific correlation has been demonstrated that was relevant to this process, until the detection of point mutations in total genomic DNA became feasible.

Experiments based on the NIH/3T3 cell transfection assay found an increased number of H-*ras* codon 12 mutations among the samples of patients with bladder cancer (8-10). This frequency ranged from 0 to 17% but the overall frequency of H-*ras* point mutations has probably been underestimated by these techniques.

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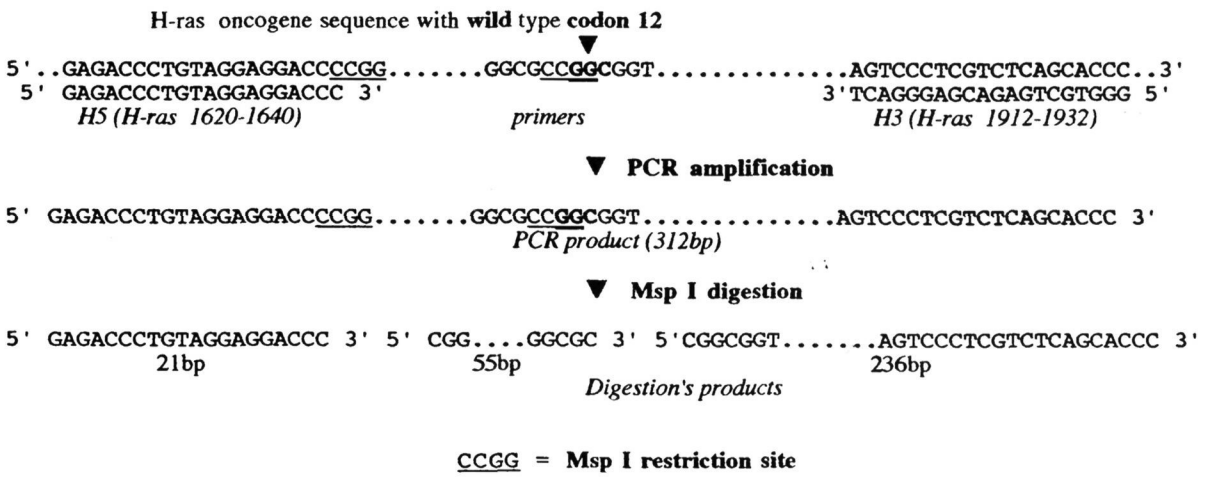

Figure 1. H-*ras* sequence and related primers.

The Polymerase Chain Reaction (PCR) is an *in vitro* method for the primer directed enzymatic amplification of specific DNA sequences. This technique allows the detection of point mutations in genes already sequenced with a sensitivity of 5% to 10% i.e. cells carrying a mutation can be detected, among a population of non-carrying cells, if they represent at least 5% of the total cell population.

However, tumors are composed of heterogeneous cell populations (neoplastic cells, infiltrated inflammatory cells, normal tissue cells). This heterogeneity increases the background 'noise' elevating the number of amplified non-mutated sequences and thus decreases the sensitivity of the detection. Therefore, it is often important to detect point mutations at this level of sensitivity in many samples of the same patient, especially after a treatment, in order to evaluate the efficiency of the therapeutic action and the level (if any) of the residual disease. The detection of a point mutation in the adjacent apparently 'normal' tissue provides a prognostic marker, relevant to the identification or the prevention of further tumor progression. These factors are of great importance in the understanding of the commitment of cells to neoplasia and malignancy.

The detection of point mutations at codon 12 of the H-*ras* oncogene into the enzymatically amplified DNA sequences, was performed by digestion of PCR products since a restriction site for the endonuclease Msp I exists in the wild type (non-mutated) form of the amplified sequence (11) but not in any mutated version. A second restriction site for Msp I was artificially introduced by the first primer in all amplified sequences (wild type and mutated) in order to be used as a control for the digestion (Fig. 1). This permitted the elimination of all incomplete digestion which could be falsely interpreted as mutated samples. The PCR amplifies a 312bp region of the H-*ras* gene, which is digested by Msp I and analysed on a 4% agarose gel. This sensitive method, connected with the feasibility of PCR amplification of DNA in cells seeded by the tumors into the urine, prompted us to evaluate the frequency of H-*ras* point mutations at codon 12 in bladder carcinomas.

Theoretically, bladder tumors should shed cells containing the H-*ras* codon 12 mutation into the urine. However, urine is a complex mixture consisting of various soluble and insoluble products, mucus, microorganisms, and contains numerous degradative enzymes derived from cells and bacteria. It was therefore unclear whether mutant genes from tumor cells could be detected in clinical specimens. In this study we have shown that H-*ras* mutations can be detected in the urine of patients with bladder tumors.

Materials and methods

Clinical specimens. Tumors, normal mucosa and urine samples were obtained from patients who underwent surgery in the Urological Clinic at Tzannion General Hospital of Pireas. Urine samples were taken one day before the operation.

DNA extraction. Tumor and mucosa samples were put directly into 300 µl lysis solution containing 0.2N NaOH/ 2M NaCl. Urine samples were centrifuged at 1,500 rpm for 10 min, the supernatant was removed and 10 µl of the concentrated cell pellet was added to the lysis solution.

All samples were boiled for 5 min and subsequently centrifuged for another 5 min. Supernatants were transferred to fresh tubes and 1 ml of ethanol was added. Tubes were stored at -20°C overnight. DNA was recovered by centrifugation for 15 min at 4°C, washed twice with 70% ethanol and resuspended in 20 µl distilled water.

Oligonucleotides and PCR amplification. The oligonucleotides (H5 and H3) used as primers for the PCR amplification of the region of the H-*ras* oncogene which includes codon 12, have been previously described (11) and are schematically presented in Fig. 1 along with the H-*ras* sequence. We also used oligos especially designed for the detection of the K-*ras* 12 mutations (12).

1 µl of DNA extract was amplified in a volume of 100 µl containing 10 mM Tris-HCl pH 8.3 / 50 mM KCl /1 mM $MgCl_2$ 200 mM dNTPs, 50 ng of each primer and 2.5 U of Taq polymerase. Amplification was performed in a Perkin Elmer Cetus Thermal Cycler using the following conditions: denaturation 1 min at 95°C, primer annealing and extension 1 min at 68°C for 35 consecutive cycles.

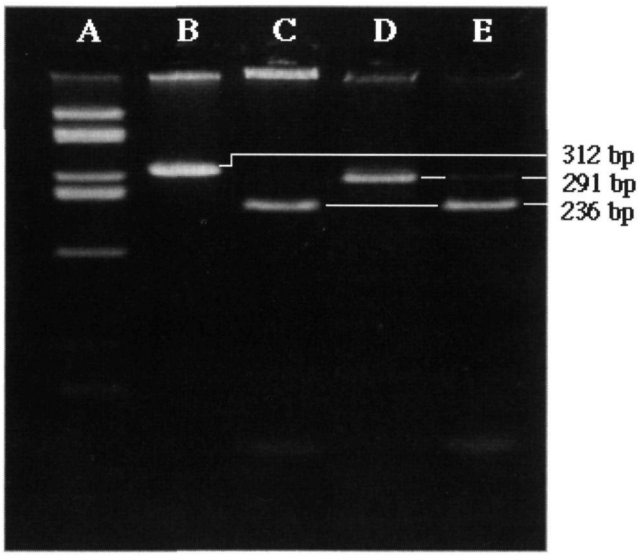

Figure 2. PCR products electrophoresis: 5-20 µl of PCR products were digested with MspI and the digestion products were electrophoresed in a agarose gel. Lane A: pUC18 / Hae III molecular weight marker. Lane B: undigested PCR product. Lane C: negative sample. Lane D: positive control (2 mutated alleles). Lane E: positive sample.

Restriction enzyme analysis. 5-20 µl of PCR products were digested with MspI at 37°C in the conditions recommended by the manufacturer of the enzyme. Digestion products were electrophoresed in a 4% LMP agarose gel containing 0.5% ethidium bromide. Gels were photographed on a UV light transilluminator.

Results and Discussion

We examined urine from 21 individuals with bladder cancer before any treatment, and tissue specimens from their excised tumor and the adjacent normal mucosa. A representative photograph of PCR products electrophoresis is shown in Fig. 2.

We found 10 mutations of the H-*ras* gene at codon 12 in the urine (47.61%), 14 mutations in the tumor specimens (66.66%) but no mutations were found in normal mucosa, as expected. These results together with patient's age, therapy, tumor primary grade and stage are presented in Table I. Fig. 3 shows a graphical representation of our findings concerning H-*ras* codon 12 mutation in bladder tumors. As shown, we can detect nearly 50% of the patients with bladder tumors using this method, which is based on the molecular pathogenesis of the disease.

The elevated frequency of the detection of mutated H-*ras* oncogene in the urine can be explained by the fact that bladder tumors contribute a significant fraction of the human DNA present in the urine as their cells turn over at an

Table I. Detection of H-*ras* codon 12 mutations in 21 patients with bladder tumors.

Patient No.	Age	Primary grade	Primary stage	Therapy	Complementary therapy	*ras* mutations detected in		
						urine	tumor	mucosa
1	63	II	$T_1 N_0 M_0$	TUR T[a]		+	+	-
2	75	II	$T_1 N_0 M_0$	TUR T		+	+	-
3	68	III	$T_1 N_0 M_0$	TUR T	epirubicin (topical)	-	-	-
4	69	II	$T_1 N_0 M_0$	TUR T	epirubicin + interferon	-	-	-
5	73	II	$T_1 N_0 M_0$	TUR T	epirubicin (topical)	+	+	-
6	70	III	$T_1 N_0 M_0$	TUR T	epirubicin (topical)	-	-	-
7	63	III	$T_3 N_0 M_0$	TUR T	irradiation	-	-	-
8	65	II	$T_2 N_0 M_0$	TUR T	irradiation	+	+	-
9	62	II	$T_1 N_0 M_0$	TUR T	epirubicin (topical)	+	+	-
10	53	II	$T_2 N_0 M_0$	TUR T		+	+	-
11	80	II	$T_1 N_0 M_0$	TUR T		-	-	-
12	73	III	$T_1 N_0 M_0$	TUR T		-	+	-
13	64	III	$T_1 N_0 M_0$	TUR T		-	+	-
14	67	II	$T_1 N_0 M_0$	TUR T	epirubicin (topical)	+	+	-
15	86	III	$T_1 N_0 M_0$	TUR T		-	+	-
16	72	III	$T_2 N_0 M_0$	TUR T	irradiation	-	-	-
17	59	II	$T_1 N_0 M_0$	TUR T		-	+	-
18	68	III	$T_2 N_0 M_0$	TUR T		+	+	-
19	90	I	$T_2 N_0 M_0$	TUR T		+	+	-
20	73	II	$T_1 N_0 M_0$	TUR T		-	-	-
21	49	II	$T_1 N_0 M_0$	TUR T	epirubicin (topical)	+	+	-

[a]TURT = transurethral resection treatment.

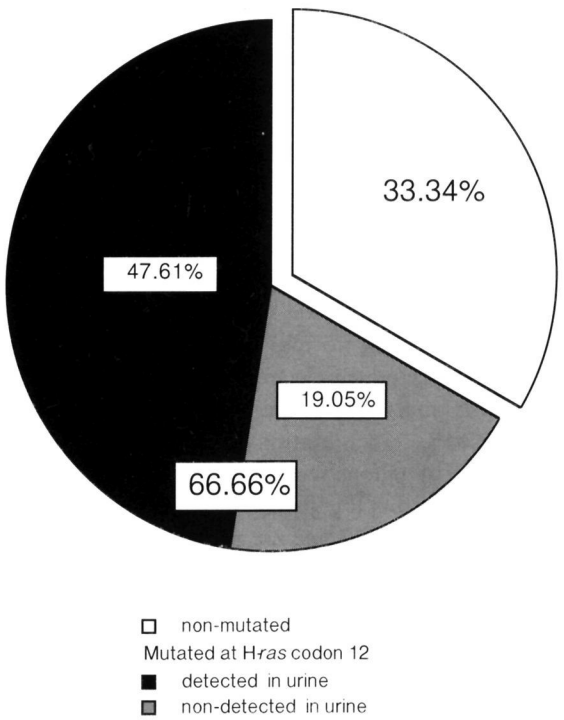

Figure 3. H-*ras* codon 12 mutations in bladder tumors.

elevated rate compared to normal cells and by the high sensitivity of the detection method. Similar results concerning the detection of mutated K-*ras* oncogene in the stool of patients with curable colorectal tumors have been reported recently (13). No mutations of the codon 12 in the K-*ras* oncogene were found in the tumors of our 21 patients with bladder carcinomas in accordance with previous findings (10).

We also studied two relapses of the patients 14 and 15. In patient 14 (which presented the mutation in the original tumor and the urine) the relapse grade had progressed from II to III and was also positive for this H-*ras* codon 12 mutation. The relapse grade for patient 15 was III and it presented for the first time the studied mutation in the urine.

Investigation into *ras* and other oncogenes and additional studies in which the urine from a large number of patients with bladder tumors of varying size, stage and relapse are studied, will be needed to determine more accurately the correlations between the detection of the mutated H-*ras* oncogenes and the invasiveness of the tumors.

These results provide the theoretical and technical basis for the detection of bladder tumors with a non-invasive method. This could eventually be used in the screening of asymptomatic patients especially those at risk from environmental factors for the presence of bladder neoplasia as well as possibly in other tumors such as head and neck, stomach, kidney, prostate, etc by appropriate adaptation of this technique to search for mutated DNA in urine or other biological fluids or excretions.

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