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Immunocytochemical Detection of p53 in Cultures of Exfoliated Cells from Urine of Patients with Urothelial Cancers

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Early diagnosis of urothelial cancer is critical for successful treatment. Mutation of the p53 gene together with allelic loss of chromosome 17p correlates well with high grade and invasiveness of urothelial cancer. Moreover, this mutation is reported to be an early event for carcinoma *in situ* of the urothelium. In order to develop a new non-invasive diagnostic method for urothelial cancer, we have established a short-term culture system for urinary exfoliated cells from patients with urothelial cancer. Immunocytochemical detection of p53 in these urine-derived cells was conducted. Short-term cultures of exfoliated cells from 50 ml samples of urine from 52 patients with urothelial cancers were made. Adequate cell growth ($>10^5$ cells per flask) was followed by passage onto glass chamber slides for p53 immunocytochemical staining. Successful passage was obtained in 40 of the 52 (76.9%) patients with urothelial cancers studied. The success rate for patients with tumors immunohistochemically positive for p53 nuclear accumulation was 90.5%, and 61.3% for those with tumors negative for p53 ($P<0.05$). Results of immunochemical analyses of the p53 in the urine cells and those in the tumor samples were identical in 92.1% of the patients. Culture of exfoliated cells from urine would be a good, non-invasive method for the molecular diagnosis of urothelial cancer that should prove useful for the early detection and follow-up of tumors with p53 mutation.

Key words: Urothelial cancer — Exfoliated cells in urine — Short-term culture — p53 — Immunocytochemistry

Urinary exfoliated cells are an important resource for non-invasive diagnosis of urothelial cancers. Positive urine cytology findings invariably indicate the presence of transitional cell carcinoma somewhere in the urinary tract, which may predate the development of discernible cancer by months or years. The sensitivity of urine cytology, however, is not satisfactory for the early detection of urinary tract tumors. We[1, 2] and others[3–5] have reported that mutation of the p53 gene occurs in approximately 50% of urothelial cancers and is strongly associated with a high grade and stage of tumor. Aberration of this gene is therefore considered a good molecular marker for urothelial cancer, in particular for tumors with high malignant potential. We previously tried immunostaining of p53 directly from smears of urinary sediment cells, but it was often difficult to diagnose p53 status due to nonspecific attachment of antibodies to the sediments. Herz *et al.*,[6] on the other hand, showed that urine from patients with bladder tumors contained viable tumor cells that could be grown *in vitro* and that these cells were of urothelial origin. We speculated that such cultures could be used to identify potential immunocytochemical tumor markers and to detect gene alterations in cultures of cells obtained from patients by non-invasive means.

To examine whether aberration of the tumor suppressor p53 can be accurately diagnosed by using cultured urine sediment cells, we conducted short-term cultures of exfoliated cells taken from the urine of patients with urothelial cancers and conducted p53 immunocytochemical analysis of these cells.

MATERIALS AND METHODS

Patients and samples Urine samples were obtained before surgery by spontaneous micturition or catheterization from 65 patients (aged 36 to 92 years, 43 men and 12 women) with urothelial cancers. The tumors consisted of 16 renal pelvic or ureteral tumors and 49 bladder tumors. Histological examinations showed that all the patients had transitional cell carcinomas (TCC). The histopathological grades (G) and stages (T) based on the TNM classification[7] were G1 (n=14), G2 (n=32), G3 (n=19), and Tis (n=11), Ta (n=26), T1 (n=12), T2 (n=6), T3 (n=9), T4 (n=1). Tis consisted of 5 primary carcinoma *in situ* (CIS) and 6 secondary CIS. Urine samples were obtained by spontaneous micturition from 47 healthy volunteers who showed normal urinalysis tested with a dipstick and had no history of urological disease (aged 25 to 85 years, 35 men and 12 women) as controls.

Short-term culture of exfoliated cells from urine Urine

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samples were collected from the second morning intermediate voiding during hospitalization or from the catheterization before cystoscopy in sterile containers. A total of 50 ml of urine was centrifuged as soon as possible in 50 ml tubes for 10 min at 500*g*. When this could not be done immediately, samples were stored for up to 1 h at 4°C. The sediments were washed twice with 10 ml of Roswell Park Memorial Institute medium containing 20% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml), after which they were transferred to 15 ml tubes, and centrifuged again. The supernatant was discarded, and the urine sediments were resuspended in 5 ml of medium and inoculated to 25 cm^2 canted-neck flasks (Corning, Cambridge, MA). Cultures were incubated at 37°C in 5% CO_2 in air. The medium was changed every 2 to 3 days. Adequate growth ($>10^5$ cells per flask) took 14 to 28 days, then the cells were transferred to 4-well glass chamber slides (Nunc, Inc., Naperville, IL) as follows. The medium was discarded, then the cells were washed with phosphate-buffered saline (PBS) and incubated for 5 to 7 min at 37°C with 1.5 ml of 0.05% trypsin, 0.02% EDTA mixture. The dispersed cells were suspended in 3.5 ml of medium then transferred to 15 ml tubes and centrifuged at 500*g* for 5 min. The sedimented cells were resuspended in 1 to 3 ml of medium, and 1 ml samples were inoculated into individual wells of chamber slides. After incubation for 2 to 4 days, immunocytochemical analysis was conducted.

p53 Immunocytochemistry The cultured cells in the chamber slides were washed with PBS, air-dried for 60 min, fixed with 10% neutral-buffered formalin for 60 min, then rehydrated in 70% ethanol. The labelled streptoavidin biotinylated immunoperoxidase method was used for immunocytochemical staining. Endogenous peroxidase activity was blocked by immersing the sections for 5 min in absolute methanol with 3% hydrogen peroxide, nonspecific binding being inactivated for 10 min with 5% skim milk. The cells were next incubated for 60 min at room temperature with anti-p53 mouse monoclonal antibody (mAb) PAb 1801 (Oncogene Science, Inc., Uniondale, NY) at the dilution of 1:100. Biotinylated antiserum was applied for 10 min at room temperature, then streptoavidin peroxidase for 10 min. Diaminobenzidine was the chromogen used to detect peroxidase activity after the 1.5 to 3 min antibody-antigen reaction. Nuclei were lightly counterstained with hematoxylin.

p53 Immunohistochemistry Surgical specimens of urinary tract tissue containing urothelial tumors were fixed with 10% neutral-buffered formalin and embedded in paraffin using the standard method. Sections of 3 μm were cut, placed on silanized glass slides, and allowed to dry overnight. These sections were deparaffinized in xylene then rehydrated in a graded series of ethanol. Before immunohistochemical staining, the deparaffinized sections were placed in a plastic jar filled with PBS and autoclaved once for 10 min at 121°C. The immunohistochemical staining method used is described in the immunocytochemistry section.

Definition The positive control for p53 immunostaining consisted of cultured DU145 cells established from a prostate cancer bearing a missense mutation of the p53 gene.[8] As the negative control, PBS was substituted for the primary antibody in the staining of the DU145 cells.

The immunostaining results were evaluated independently by two investigators (H.O. and Y.K.). Tumors in which more than 10% of the cells exhibited intense and homogeneous or heterogeneous nuclear reactivity were classified as positive staining according to the reports by Esrig and associates.[4, 9] Cytoplasmic reactivity and scattered staining were classified as negative staining. As there has been no other report on p53 staining of urinary sediment cells, we arbitrarily defined 10% as the cut-off for p53 positivity.

Statistical analysis Student's t test or the χ^2 test was used, for examination of the significance of differences, the criterion of significance being $P<0.05$.

RESULTS

Success rate of short-term culture Sixty-five patients with urothelial cancers and 47 healthy volunteers initially were included in the study. Because of contamination by bacteria, fungi, or both, samples from 13 (20.0%) patients and 4 (8.5%) volunteers were excluded. Results for the remaining 52 patients and 43 volunteers were analyzed. Successful primary cell outgrowth occurred in 48 (92.3%) of the 52 patients, and in 27 (62.8%) of the healthy volunteers ($P<0.05$). The average periods required for primary cell outgrowth from the urine of the patients and volunteers respectively were 5.8±1.6 and 8.0±2.9 days and for successful passage, 21.9±4.6 and 26.0±3.4 days (data not shown). Forty of the 52 (76.9%) patients and 15 of the 43 (34.9%) healthy volunteers had successful passages, the difference in the success rates being significant ($P<0.0001$).

Nuclear accumulation of the p53 protein in surgical specimens from the 52 patients was analyzed immunohistochemically. p53-Positive tumors were found in 21; the tumor grades and pathological stages are shown in Table I. Interestingly, the rate of successful passage for the urine cell samples from patients with p53-positive tumors (19/21, 90.5%) was significantly higher than that for patients with p53-negative tumors (20/31, 61.3%) ($P<0.05$) (Table I).

Correlation of positive p53 immunostaining for cultured cells and the corresponding tumors Cultured exfoliated

Table I. Correlation of Pathological Parameters or Success Rate of Culture with p53 Status in Surgical Specimens

p53 Immunohistochemical staining of surgical specimens	No. of patients	Grade			T-stage							No. of successful primary outgrowths	No. of successful passages
		1	2	3	is	a	1	2	3	4			
(+)	21	2	12	7	3	5	5	2	6	0		21 (100%)	19 (90.5%)[a]
(−)	31	10	14	7	6	16	5	2	1	1		26 (83.9%)	20 (61.3%)

a) Significantly different from p53 (−) by χ^2 test, $P<0.05$.

Table II. Comparison of p53 Results of Immunocytochemical Staining of Cultured Urine Cells and Immunohistochemical Staining of a Surgical Specimen

Immunocytochemistry in cultured urine cells	Immunohistochemistry in surgical specimen		Total
	p53 (+)	p53 (−)	
p53 (+)	17	1	18
p53 (−)	2	18	20
	19	19	38

cells from urine samples of 38 of 40 patients with urothelial cancers and 7 of 15 healthy volunteers with successful passages were prepared for p53 immunocytochemical analyses. The others were taken for cytokeratin-18 immunocytochemical or Papanicolaou staining (data not shown). Cells with nuclear accumulation of p53 protein in numbers corresponding to more than 10% of the growing cells (Fig. 1A) were observed in 18 (47.4%) of the patients, whereas there were no such cells in the healthy volunteers (Fig. 1B). The urine samples and tumors of 17 patients showed identical p53 nuclear accumulations, but urine samples from 2 patients were negative for p53 in spite of positive tumor staining (sensitivity: 89.4%). For the other 20 patients whose urine cells were negative for p53 staining, the tumors of 18 were also negative for p53 (specificity: 94.7%). Consequently, a comparison of the p53 immunostaining status of the cultured cells from the urine and those of tumor tissue obtained from individual patients showed they were identical in 35 of 38 patients (92.1%) (Table II).

We tried more than one passage in several patients. In 9 of these patients, culture of the urine cells could be maintained for 3 or more passages. Immortalization, however, has not been achieved in any of these cells (the maximal passage number was 6). Seven of these 9 patients had tumors with nuclear accumulation of p53 and an increased percentage of p53-positive cells in urine culture was clearly recognized in two p53-positive tumor cases (Fig. 2).

DISCUSSION

Our findings clearly show that human urine contains viable cells which can be propagated *in vitro*. These cells appear to be exfoliated from the interior surface of the urinary tract, as an immunocytochemical study showed that they were strongly stained with anti-cytokeratin-18 mouse mAb (unpublished data), indicative of their having an epithelial origin.[10] Moreover, electron microscopy by others has shown that cultured cells from urine form desmosomes and tight junctions and are lined with the asymmetric unit membrane characteristic of the urothelium.[11] Cells from normal urothelium, as well as those from urothelial tumors, can be propagated *in vitro,* because adequate cell growth was obtained with cells from the urine of healthy volunteers, even though the overall success rate for the growth of cells from non-cancerous subjects was significantly lower. The increase in the percentage of p53-positive cells during successful passages indicates that cells exfoliated from tumors are more prone to *in vitro* growth than are those of normal urothelial origin.

Early diagnosis of urothelial cancer is critical for successful treatment. We[1,2] and others[3–5] have reported that mutation of the p53 gene correlates well with the grade and invasiveness of urothelial cancers. Moreover, the nuclear accumulation of p53 corresponds well with mutation of the p53 gene, which results in elongation of the half-life of the molecule,[4] although there are a few exceptions.[12] Esrig *et al.* reported that the nuclear accumulation of p53 is closely associated with tumor recurrence.[9] Furthermore, p53 mutation is reported to be an early event for CIS of the urothelium.[13] CIS in the urothelium often manifests progression to invasive cancer, but accurate diagnosis is not easy. We therefore used abnormal nuclear accumulation of p53 as a molecular marker to examine whether this urine culture system can provide the basis of a new non-invasive method for the diagnosis of urothelial cancers, especially those with high risk of progression.

Previously, we conducted an immunocytochemical analysis of p53 directly from smears of urine sediment cells, but it was often difficult to detect p53 due to nonspecific attachment of antibodies to the sediment

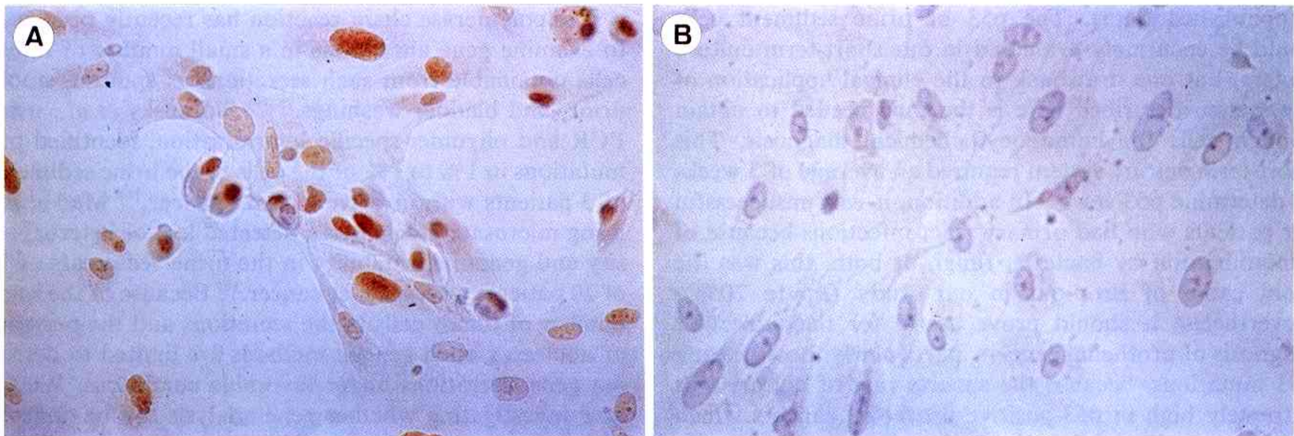

Fig. 1. Immunocytochemical staining for p53 in cultured urine-derived cells. (A) Nuclear accumulation of p53 is observed in approximately 50% of the cells in a patient with p53-positive tumor (TCC, G2, pT1 No Mo) in the renal pelvis (×130). (B) No nuclear accumulation of p53 in the cells from urine of a 43-year-old healthy volunteer (×130).

Fig. 2. p53-Immunostaining for a 67-year-old patient with bladder tumor (TCC, G2, pT1 No Mo). (A) p53-Positive staining of the surgical specimen (×130). (B) p53-Positive staining (15%) in the cultured urine-derived cells at the first passage (×130). (C) p53-Positive staining (40%) in the cultured urine-derived cells at the third passage (×130).

(unpublished data). The p53 of urine sediment cells could be accurately evaluated in our short-term culture system, but one drawback to the clinical application of the system described here is the time needed to obtain enough cells for immunocytochemical diagnosis. This short-term culture system required an average of 3 weeks to determine p53 status. In addition, it was unsuccessful for patients who had urinary tract infections because of contamination by bacteria, fungi, or both; this was the main cause of drop-out in our study (up to 20%). Nevertheless it should prove useful for the molecular diagnosis of urothelial cancers, particularly those bearing p53 mutations, because the success rate of culture was extremely high in p53-positive urothelial cancers which require intensive follow-up, and it is non-invasive. To shorten the time necessary for diagnosis, we recently tried direct inoculation and cultivation of urine sediment cells into 1-well glass chamber slides. This alternative method was successful in 11 of 13 patients and the results can be obtained within 10 days. Another concern in this system is the possibility of false-positive diagnosis due to the existence of apoptotic normal cells.[14] We, however, consider this unlikely, because increased nuclear expression of p53 was never observed in cultured cells from any of the 7 healthy volunteers. Although the results of p53 staining for tumors and urine cultured cells showed good coincidence, urine cells from 2 patients with p53-positive tumors were negative for p53 staining. As there has been no other report on p53 staining of urinary sediment cells, we have arbitrarily defined 10% as the cut-off for p53 positivity, but an appropriate cut-off value of p53 staining for urine cultured cells remains to be decided.

The polymerase chain reaction has recently been used to examine gene alterations in a small number of tumor cells obtainable from such secretions as sputum, stools, urine, and bladder washings.[15-19] Sidransky *et al.*, using PCR and oligomer-specific hybridization, identified p53 mutations in 1% to 7% of the cells in the urine sediments of 3 patients with invasive bladder cancer.[16] Mao *et al.*, using microsatellite analysis, detected loss of heterozygosity and genomic instability in the urine sediments of 19 of 20 patients with bladder cancer.[18] Because of the small number of tumor cells in the secretions and the presence of nucleases, such genetic methods are limited to detecting gene alterations under favorable conditions. We are now investigating whether gene analysis can be done on DNA obtained from the proliferating cells in our system. Using mutant allele-specific amplification methods,[20,21] we detected an identical p53 gene mutation in cultured cells obtained from a patient who had bladder cancer in which there was a point mutation of the p53 gene (data not shown). These findings indicate that this culture system should also provide a good source of cells for the genetic analysis of urothelial cancer by non-invasive means.

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