[CANCER RESEARCH 59, 3606–3609, August 1, 1999]

Specific *p53* Gene Mutations in Urinary Bladder Epithelium after the Chernobyl Accident[1]

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ABSTRACT

After the Chernobyl accident, the incidence of urinary bladder cancers in the Ukraine population increased gradually from 26.2 to 36.1 per 100,000 between 1986 and 1996. Urinary bladder epithelium biopsied from 45 male patients with benign prostatic hyperplasia living in radiocontaminated areas of Ukraine demonstrated frequent severe urothelial dysplasia, carcinoma *in situ*, and a single invasive transitional cell carcinoma, combined with irradiation cystitis in 42 cases (93%). No neoplastic changes (carcinoma *in situ* or transitional cell carcinoma) were found in 10 patients from clean areas (areas without radiocontamination). DNA was extracted from the altered urothelium of selected paraffin-embedded specimens that showed obviously abnormal histology (3 cases) or intense p53 immunoreactivity (15 cases), and mutational analysis of exons 5–8 of the *p53* gene was performed by PCR-single-strand conformational polymorphism analysis followed by DNA sequencing. Nine of 17 patients (53%) had one or more mutations in the altered urothelium. Urine sediment samples were also collected from the patients at 4–27 months after biopsy and analyzed by PCR-single-strand conformational polymorphism analysis or yeast functional assay, and identical or additional *p53* mutations were found in four of five cases. Interestingly, a relative hot spot at codon 245 was found in five of nine (56%) cases with mutations, and 11 of the 13 mutations determined (73%) were G:C to A:T transitions at CpG dinucleotides, reported to be relatively infrequent (~18%) in human urinary bladder cancers. Therefore, the frequent and specific *p53* mutations found in these male patients may alert us to a future elevated occurrence of urinary bladder cancers in the radiocontaminated areas.

INTRODUCTION

Due to the Chernobyl Nuclear Power Plant accident in April 1986, more than 10 million people are currently living in radiocontaminated areas of Ukraine, Belarus, and Russia. They have been exposed for more than 12 years to low doses of ionizing radiation. ^{137}Cs and, to a lesser extent, ^{134}Cs constitute 80–90% of the incorporated radioactivity in people living in these areas, and these radionuclides are known to be concentrated and excreted in the urine (1). In the 11 years between 1986 and 1996, the incidence of urinary bladder cancer in the Ukraine population increased from 26.2 to 36.1 per 100,000 (2). A significant increase in childhood thyroid cancer was also observed a few years after the Chernobyl accident (3, 4); *p53* gene mutations were reported to be infrequent (5), but rearrangements of the *ret* oncogene were frequent (6) in these patients.

Mutational inactivation of the *p53* tumor suppressor gene is one of the most common genetic alterations found in human cancers (7). In the case of the urinary bladder, it has been reported that *p53* mutations are common in invasive and/or high-grade tumors, and roles in dedifferentiation or tumor progression have therefore been speculated (8–10). Spruck *et al.* (11) have suggested the participation of two molecular pathways in urinary bladder carcinogenesis, with *p53* alterations occurring early in CIS[3] and dysplasia before the development of nonpapillary invasive lesions but occurring late in papillary TCCs. Thus, early detection of *p53* mutations in urinary bladder epithelial lesions may be strongly predictive of future urinary bladder cancer, especially that of the nonpapillary invasive type. Recently, we histologically investigated the urinary bladder epithelium of patients living in radiocontaminated areas of Ukraine who received a transbladder prostatectomy due to BPH (12). Although they were all without symptoms of urinary bladder disease, severe urothelial dysplasia and/or CIS with concomitant irradiation cystitis were extraordinaly frequent in these patients (12). In the present study, the biopsied urinary bladder specimens were analyzed for mutational inactivation of the *p53* gene by PCR-SSCP analysis. Moreover, urine sediments collected after an interval were examined by PCR-SSCP and the yeast functional assay (13). The yeast functional assay tests the ability of human p53 to activate transcription in yeast; colonies containing wild-type p53 are white, whereas these containing mutant p53 are red. Because human *p53* cDNA PCR products can be cloned directly into the reporter yeast strain by homologous recombination without intermediate bacterial cloning steps, the percentage of red yeast colonies accurately reflects the mutant *p53* mRNA content of the starting material. Therefore, the assay can detect a mutant *p53* in a minor fraction of cell clones such as those in urine sediments.

MATERIALS AND METHODS

Patients. The subjects were 55 male patients (49–92 years old) undergoing surgery for BPH at the Institute of Urology and Nephrology, Academy of Medical Sciences of Ukraine (Kiev, Ukraine) between 1994 and 1997. All 55 patients received multiple mapping biopsies of the urinary bladder mucosa during the operation for BPH. The group I patients (28 of 55 patients; average age, 62 years) had been continuously inhabiting radiocontaminated areas of Ukraine with the density of ^{137}Cs contamination of $\geq$5–30 Ci/km^2, and group II patients (17 of 55 patients; average age, 75 years) were from Kiev city (^{137}Cs contamination, 0.5–5 Ci/km^2; Ref. 14). The group III controls were 10 patients (average age, 66 years) living in so-called "clean" areas of the country (areas without radiocontamination; Ref. 14). Although detailed information was not available, the majority of patients had smoked for more than 20 years (about 20 cigarettes/day).

Histological Examination. Formalin-fixed, paraffin-embedded tissue blocks were routinely processed, sectioned, and stained with H&E for histological examination. Before molecular analysis, all urothelial lesions (severe dysplasia, CIS, or small invasive TCC) were immunohistochemically investigated with an anti-p53 antibody (DO-7; DAKO, Glostrup, Denmark; Ref. 15) and assessed as described previously (12).

DNA Preparation. Urinary bladder epithelial lesions with intensive p53 nuclear immunoreactivity (>10% of cells stained) or without positivity for p53 but with histological abnormalities (severe dysplasia, CIS, or TCC) were

Received 11/13/98; accepted 6/2/99.

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[1] Supported by funds from the Project of Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation.

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[3] The abbreviations used are: CIS, carcinoma *in situ*; TCC, transitional cell carcinoma; BPH, benign prostatic hyperplasia; SSCP, single-strand conformational polymorphism.

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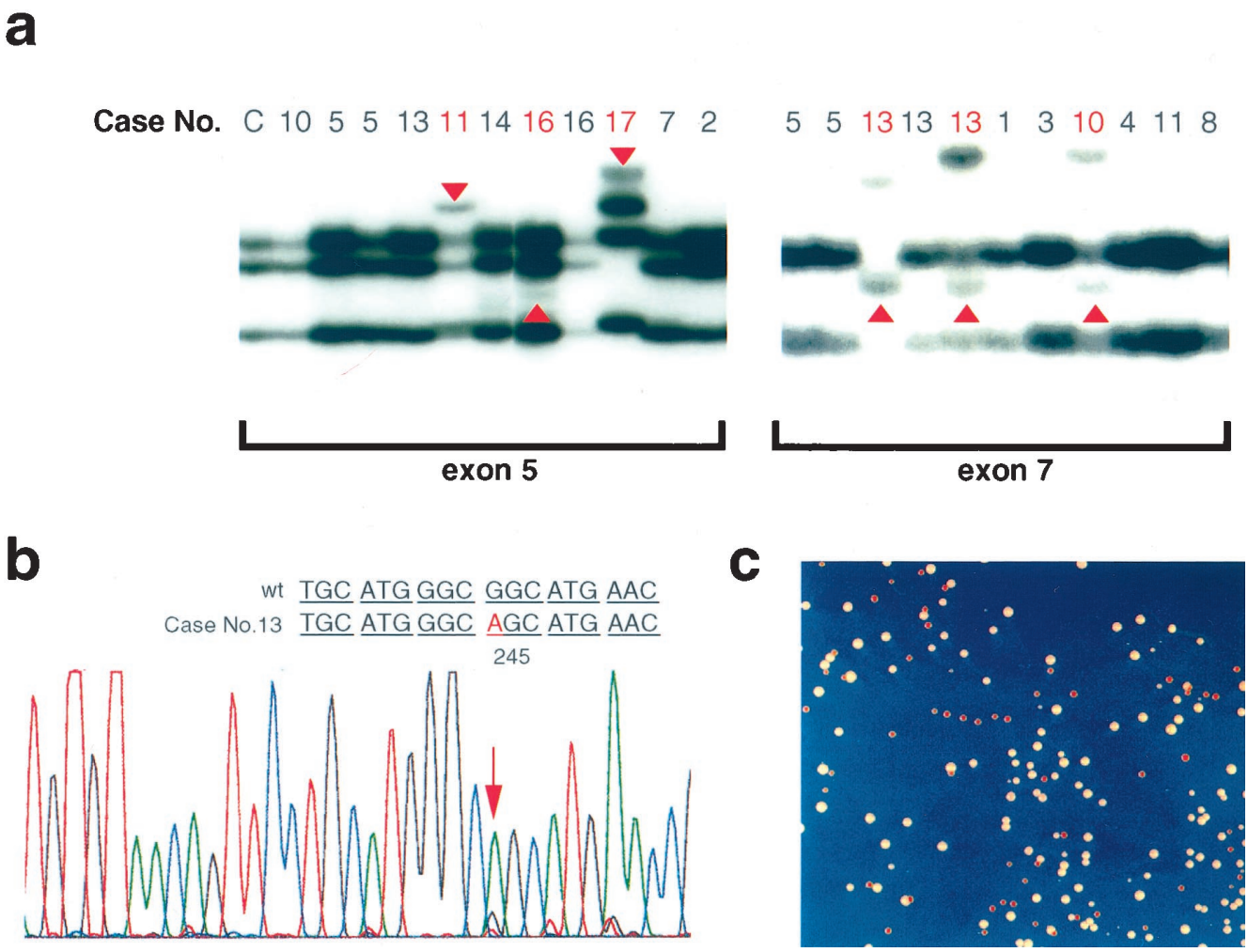

Fig. 1. Results of mutational analyses of the *p53* gene for lesions in patients living in radiocontaminated areas of Ukraine. *a*, PCR-SSCP analysis of *p53* gene exons 5 (*left panel*) and 7 (*right panel*) using DNAs prepared from altered urothelium. Case numbers with mutations are shown in *red*. Case *C* is human genomic DNA (Promega) used as a control. *b*, sequence analysis of case 13 revealed a GGC (Gly) to AGC (Ser) transition mutation at codon 245. *c*, yeast functional assay of case 17 gave 29% red colonies.

selected for DNA extraction. DNA for PCR was prepared from paraffin-embedded sections using a microdissection approach, as described previously (16). Briefly, serial sections adjacent to those used for histological analysis were prepared at a thickness of 3–7 μm, deparaffinized, and air-dried. Using a fine needle, selected epithelial lesions (length, 3–8 mm) were dissected out under a microscope. Tissues were collected in 20–100 μl of protein lysis buffer containing 0.1 mg/ml proteinase K. After adequate digestion, proteinase K was inactivated by boiling, and samples were diluted to an optimized concentration for PCR. For the samples with sufficient tissue, a part of the solution after digestion with proteinase K underwent DNA extraction with a kit (Sepagene; Sankyo Junyaku Co., Tokyo, Japan). The resulting DNA pellets were diluted with distilled water for PCR. Finally, 21 samples from 15 patients of groups I and II were available for analysis.

PCR-SSCP and Direct Sequencing. For the mutational analysis of *p53* gene exons 5–8, PCR-SSCP analysis (17) and direct sequencing were performed using the procedures described previously (18), with minor modifications. Primer sequences used were as follows: (*a*) exon 5, 5′-TTCAACTCTGTCTCCTTCCT-3′ and 5′-CAGCCCTGTCGTCTCTCCAG-3′; (*b*) exon 6, 5′-GCGTCTGATTCCTCACTGAT-3′ and 5′-TTAACCCCTCCTCCCAGAGA-3′; (*c*) exon 7, 5′-AGGCGCACTGGCCTCATCTT-3′ and 5′-TGTGCAGGGTGGCAAGTGGC-3′; and (*d*) exon 8, 5′-TTCCTTACTGCCTCTTGCTT-3′ and 5′-AGGCATAACTGCACCCTTGG-3′. To eliminate nonspecific amplification, hot start PCR was applied using AmpliTaq Gold (Perkin-Elmer Cetus Instruments, Norwalk, CT) according to the manufacturer's instructions. PCR including [^{32}P]dCTP for SSCP analysis was carried out under the following conditions: initial preheating at 96°C for 10 min to achieve enzymatic activity; followed by 38 reaction cycles (96°C for 30 s, annealing temperature varied between 54°C and 58°C for 30 s and 72°C for 30 s) and a final elongation (72°C for 12 min). In all cases with mutation, PCR-SSCP analysis was repeated at least once using independent PCR products, and the existence of a mutation was confirmed by direct sequencing. Throughout the experiment, special care was taken to avoid contamination of template DNA. PCR reagents were kept physically separated from the areas where PCR products were handled, and reagents were mixed in a COY Template Tamer hood (COY Co., Grass Lake, MI) equipped with UV light. For some cases with mutations, corresponding normal prostate or lymphatic tissues were included for analysis to test the presence of constitutional polymorphisms and germ-line mutations.

Assessment of Urine Samples. At 4–26 months after the biopsy, urine sediments were collected from six patients as described by Sidransky *et al.* (8), immediately frozen, and stored until use. Nucleic acids were extracted from pellets using Isogen (Nippon Gene, Toyama, Japan); the DNA layer was then further treated with Sepagene (Sankyo Junyaku). Sufficient amounts of RNA were obtained from two cases (cases 6 and 17) and used for p53 yeast functional assays as described previously (13). DNA of urine sediment was available for three cases (cases 12, 14, and 15) and analyzed by PCR-SSCP as described above.

Statistical Analysis. Differences in the proportions of mutation patterns were examined for statistical significance with the χ^2 test.

RESULTS

In groups I and II, all cases exhibited proliferative cystitis, *i.e.*, von Brunn's nests, cystitis cystica, and squamous and glandular metaplasias, that were frequently combined and had features of irradiation

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cystitis rather than simple inflammation. Multiple areas of severe dysplasia were detected in 42 of 45 (93%) patients, and 22 of 45 patients (49%) demonstrated areas of CIS. On the other hand, no neoplastic changes were found in the group III urothelium, although mild inflammation was evident within both the urothelium and submucosal tissues. Details of the immunohistochemical analysis of these urothelial lesions have been reported elsewhere (12). DNA was extracted from the selected areas, and PCR-SSCP analysis was performed on 21 samples from 15 cases. In addition, urine samples were assessed by PCR-SSCP analysis (three cases) and yeast functional assay (two cases). Overall mutational analyses were performed for 17 patients, with all but 3 patients (patients 1, 5, and 7) being intensively positive for p53 immunohistochemistry.

Results of mutational analysis of the *p53* gene are illustrated in Fig. 1. PCR-SSCP revealed that 9 of 17 cases (53%) harbored one or more *p53* mutations within identical or separate samples (Table 1). In three cases (cases 13–15), identical mutations were found in separate samples, and a clonal relationship was strongly suggested. Considering these mutations as single events, a total of 15 mutations were found in nine cases. All *p53* mutations determined were single-bp substitutions, and no base deletions or insertions were found. All but one mutation [case 16, codon 154; GGC (Gly) to GGT (Gly)] resulted in amino acid changes. A total of 1 (6.7%), 4 (27%), 1 (6.7%), and 9 (60%) mutations were found in *p53* exons 4, 5, 6, and 7, respectively, and no mutation was found in exon 8. Eleven of 15 (73%) mutations determined were G:C to A:T transitions at CpG dinucleotides, and relative hot spots were noted involving three CpG dinucleotides (codons 158, 245, and 248). Mutations at these sites have not been reported to be frequent in human urinary bladder cancers (19, 20). In the IARC database compiled by Hainaut *et al.* (20), G:C to A:T transitions at CpG dinucleotides account for only 18.2% of the reported 457 *p53* mutations in urinary bladder tumors, demonstrating a significant difference from our present data (χ^2 test, $P < 3.5 \times 10^{-9}$). Because 9 of 15 mutations determined were concentrated between codons 245 and 254 on exon 7, a primer pair was designed to include this region [the upstream primer (5′-ACTACATGTGTAACAGTTCC-3′) and downstream primer (5′-TCCTGACCTGGAGTCTTCCA-3′) produce an 86-bp short PCR fragment], and PCR-SSCP analysis was performed for the DNAs extracted from the urothelium of nine patients living in clean areas (group III). No abnormal bandshifts were found.

In two cases (cases 14 and 15), mutations determined in the biopsy samples were also found in the subsequent urine sediments by PCR-SSCP analysis, suggesting the presence of mutated clones in the urothelium and the exfoliation of significant numbers of altered cells into the urine. In cases 6 and 17, p53 yeast functional assays of urine samples gave 4% and 29% red colonies (Fig. 1*c*), respectively, and more than four red yeast colonies were randomly selected and sequenced in both cases. In case 6, no identical mutation was found; therefore, this case was considered negative for clonal mutation. A pair of tandem mutations was evident in case 17 (codons 125 and 211 on the same cDNA fragment), clonal in 4 of 5 colonies.

DISCUSSION

In the present study, mutational analysis of the *p53* gene in DNA extracted from the urothelium of patients living in radiocontaminated areas of Ukraine revealed that 9 of 17 cases (53%) harbored one or more mutations within identical or separated samples (Table 1). This frequency is similar to those described for human high-grade, invasive urinary bladder cancers (9, 11). Although base deletions or insertions of the *p53* gene have been found in a certain proportion of human urinary bladder cancers (19), all *p53* mutations identified in this study were single-bp substitutions. The most striking feature is the predominance of G:C to A:T transition mutations at CpG dinucleotides, especially on codons 158, 245, and 248. Although ionizing radiation has been reported to cause a variety of types of DNA damage including strand breaks and cross-linking (21), direct *in vivo* evidence

Table 1 *Mutational analysis of the p53 gene in urothelial lesions in male patients living in radiocontaminated areas of Ukraine*

						p53 mutation			
Case no.	Age (yr)/group	Date of sampling[a]	Sample	Histology	Exon	Codon	Base change	Amino acid change	Substitution
1	57/I	Feb. 1997	Biopsy	Dysplasia[b]	None				
2	69/I	May 1996	Biopsy	Dysplasia	None				
3	60/II	May 1995	Biopsy	Dysplasia	None				
4	74/I	Nov. 1996	Biopsy	CIS	None				
5	67/I	Mar. 1996	Biopsy	CIS	None				
6	78/II	May 1996	Biopsy	Dysplasia	NE[c]				
		July 1997	Urine (yeast)		None				
7	69/I	Dec. 1994	Biopsy	Dysplasia	None				
8	68/I	July 1996	Biopsy	Dysplasia	None				
9	66/I	Nov. 1994	Biopsy	Dysplasia	7	251	ATC→CTC	Ile→Leu	A→C
10	63/I	Jan. 1995	Biopsy	CIS	7	245	GGC→AGC	Gly→Ser	G→A/CpG
11	73/I	Feb. 1997	Biopsy	Invasive TCC	5	158	CGC→CAC	Arg→His	G→A/CpG
12	68/I	May 1995	Biopsy	CIS	NE[c]				
		July 1997	Urine (SSCP)		5	175	CGC→TGC	Arg→Cys	C→T/CpG
13	66/I	Mar. 1995	Biopsy	Dysplasia	7	245	GGC→AGC	Gly→Ser	G→A/CpG
			Biopsy	CIS	7	245	GGC→AGC	Gly→Ser	G→A/CpG
14	63/I	Jan. 1997	Biopsy	Dysplasia	7	245	GGC→AGC	Gly→Ser	G→A/CpG
		July 1997	Urine (SSCP)		7	245	GGC→AGC	Gly→Ser	G→A/CpG
15	68/I	July 1996	Biopsy	Dysplasia	7	245	GGC→AGC	Gly→Ser	G→A/CpG
		Aug. 1997	Urine (SSCP)		7	245	GGC→AGC	Gly→Ser	G→A/CpG
16	69/II	Feb. 1997	Biopsy	Dysplasia	5	154	GGC→GGT	Gly→Gly	C→T/non-CpG
					7	245[d]	GGC→AGC	Gly→Ser	G→A/CpG
					7	254[d]	ATC→ACC	Ile→Thr	T→C
			Biopsy	Dysplasia	7	248	CGG→TGG	Arg→Trp	C→T/CpG
17	70/I	May 1995	Biopsy	CIS	5	158	CGC→CAC	Arg→His	G→A/CpG
					7	248	CGG→TGG	Arg→Trp	C→T/CpG
		Aug. 1997	Urine (yeast)		4	125[d]	ACG→ATG	Arg→Met	C→T/CpG
					6	211[d]	ACT→ATT	Thr→Ile	C→T/non-CpG

[a] Feb., February; Nov., November; Mar., March; Dec., December; Jan., January; Aug., August.
[b] Moderate to severe dysplasia.
[c] NE, not evaluated because samples for PCR were not available.
[d] Tandem mutations on the same allele.

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of radiation-induced bp substitutions is lacking. Sikpi *et al.* (22) reported that the mutation frequencies of γ-irradiated (^{137}Cs) plasmid DNA replicated in a human lymphoblastoid cell line were increased about 62-fold over background levels, although the percentage of G:C to A:T transition mutations was not affected. As for childhood thyroid cancers after the Chernobyl accident, *p53* mutations have been shown to be infrequent, with no specific mutations apparent (5). However, *ret* rearrangement was found to be frequent (6). Thus the underlying mechanism might be different from that responsible for the specific mutations observed in this study. In human urinary bladder cancers, no specific bp substitution pattern for the *p53* gene has hitherto been described, and there has been no pointer to any specific mutagen (7, 19, 20). On the other hand, mutational analysis of schistosomal urinary bladder cancer (endemic in Egypt) gave results that are very consistent with our findings; namely, a high proportion of bp changes at CpG dinucleotides (18 of 34; 53%; Ref. 23). Chronic urinary infection with *Schistosoma hematobium* is a significant etiological factor in schistosomal bladder cancer. Irradiation cystitis was a common characteristic feature of cases in the present study. Recently, a close relationship between chronic infection and cancer risk has been suggested, with the production of nitric oxide during inflammatory processes playing a role (24). It has been shown that nitric oxide can produce transitions at CpG dinucleotides by deamination of 5-methylcytosine (24). In addition, endogenous formation of urinary *N*-nitroso compounds leads to O^6-alkylguanine formation and G:C to A:T transitions (23). To ascertain the specificity of *p53* mutations observed in the present study, we compared the mutational spectrum of urinary bladder cancers of Ukrainian patients before and after the Chernobyl accident as well as normal autopsy-derived urinary bladder mucosa.[4]

Two techniques were used in the present study to determine the *p53* gene mutations in urine samples: (*a*) PCR-SSCP analysis (17); and (*b*) p53 yeast functional assay (13). When the PCR-SSCP technique is used to analyze *p53* mutations, significant amounts of mutated cells are necessary (usually at least 20% of the total). However, if we can determine clonal and characteristic mutations in several red colonies by the yeast functional assay, it will allow the use of urine samples. We are now collecting urine samples from the general population in radiocontaminated areas of Ukraine to further assess the applicability of these noninvasive techniques.

Of the nine cases with *p53* mutations, two cases (cases 16 and 17) proved to have multiple *p53* mutations in their urinary tract, as reported previously by Spruck *et al.* (11) and Goto *et al.* (25). Different *p53* mutations in independent urothelial lesions (case 16) or in metachronous samples (case 17) indicate that a strong carcinogenic insult may have resulted in multiple transformation events in a large field of urothelium, as demonstrated previously in an animal model (16).

The frequent (9 of 17 cases, 53%) *p53* mutations of altered urinary bladder epithelium in patients who visited the hospital without symptoms of urinary bladder disease suggest that the prediction of induction of urinary bladder cancer may be possible. More precise and widely applicable screening tests are now required for the residents of radiocontaminated areas.

ACKNOWLEDGMENTS

We thank Dr. Yoshihisa Yano (Second Department of Biochemistry, Osaka City University Medical School, Osaka, Japan) for advice. We are grateful to Emi Kawakami, Kuniko Nishizawa, Mari Dokoh, and Satomi Katagiri for assistance with this experiment.

[4] Unpublished data.

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