

## Urinary Bladder Lesions after the Chernobyl Accident: Immunohistochemical Assessment of p53, Proliferating Cell Nuclear Antigen, Cyclin D1 and p21<sup>WAF1/Cip1</sup>

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During the 11-year period subsequent to the Chernobyl accident, the incidence of urinary bladder cancer in Ukraine has increased from 26.2 to 36.1 per 100,000 population. Cesium-137 (<sup>137</sup>Cs) accounts for 80–90% of the incorporated radioactivity in this population, which has been exposed to long-term, low-dose ionizing radiation, and 80% of the more labile pool of cesium is excreted via the urine. The present study was performed to evaluate the histopathological features and the immunohistochemical status of p53, p21<sup>WAF1/Cip1</sup>, cyclin D1 and PCNA (proliferating cell nuclear antigen) in urinary bladder mucosa of 55 males (49–92 years old) with benign prostatic hyperplasia who underwent surgery in Kiev, Ukraine, in 1995 and 1996. Group I (28 patients) inhabiting radiocontaminated areas of the country, group II (17 patients) from Kiev city with less radiocontamination and a control group III (10 patients) living in so-called “clean” areas of Ukraine were compared. In groups I and II, an increase in multiple areas of moderate or severe dysplasia or carcinoma *in situ* was seen in 42 (93%) of 45 cases. In addition, two small transitional cell carcinomas were found in one patient in each of groups I and II. Nuclear accumulation of p53, PCNA, cyclin D1, and to a lesser extent p21<sup>WAF1/Cip1</sup>, was significantly increased in both groups I and II as compared with the control group III, indicating possible transformation events or enhancement of repair activities, that may precede the defect in the regulatory pathway itself, at least in the G1 phase of the cell cycle. Our results suggest that early malignant transformation is taking place in the bladder urothelium of people in the radiocontaminated areas of Ukraine and that this could possibly lead sometime in the future to an increased incidence of urinary bladder cancer.

Key words: Urinary bladder cancer — Cell cycle — p53 — Cyclin D1 — p21<sup>WAF1/Cip1</sup>

Human urinary bladder cancer is well known to be associated with exposures to various environmental or occupational agents.<sup>1)</sup> About 10,000,000 people living in Ukraine and Russia have been exposed to low doses of ionizing radiation for more than 11 years since the Chernobyl accident. Cesium (<sup>137</sup>Cs and to a lesser extent <sup>134</sup>Cs) accounts for 80–90% of the internal (incorporated) radioactivity in those individuals. These radionuclides become concentrated, like potassium, in the urine during excretion. As much as 80% of the more labile pool of cesium is excreted through the urine,<sup>2,3)</sup> and therefore, carcinogenic effects on the urinary bladder may be anticipated. During the 11-year period (1986–1996) after the Chernobyl accident, the incidence of urinary bladder cancer in Ukraine has increased from 26.2 to 36.1 per 100,000 individuals and the morbidity from 6.7 to 10.2 per 100,000.<sup>4)</sup>

Theoretically, a cancer risk at high doses of ionizing radiation implies that low doses also constitute a hazard.<sup>5)</sup>

However, no publication dealing with urinary bladder urothelial lesions of people with long-term exposure to low doses of ionizing radiation has appeared. The possibility that molecular genetic changes might be present in human bladder urothelium of apparently healthy people living in radiocontaminated areas has also not yet been explored.

In multi-stage carcinogenesis of the urinary bladder cancer, early genetic alterations are considered to represent the initial steps towards transformation of urothelial cells.<sup>6)</sup> A prolonged increase in cell proliferation is also important for the development of tumors in man.<sup>6,7)</sup> The development and progression of tumors are generally driven by an accumulation of genetic alterations.<sup>8)</sup> A pivotal role of *p53* mutation (usually involving exons 5–8) in bladder carcinogenesis has been shown by investigations of human and experimental materials.<sup>9–12)</sup> In addition, several recent papers have indicated that *p53* might be a critical “marker gene” of the cellular response to ionizing radiation.<sup>13)</sup> In the last few years, a link between p53 protein and the proliferating cell nuclear antigen (PCNA) has

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been identified.<sup>14</sup> Differences in the staining pattern and intensity of PCNA between mild and moderate dysplasia and various papillary tumors of the urinary bladder suggests the usefulness of this antigen as a "marker" of DNA synthesis and cell proliferation in bladder neoplasm diagnostics.<sup>15, 16</sup>

Cyclin D1, in cooperation with its cyclin-dependent kinase (CDK) major catalytic partners, plays an important role in regulating the progression of cells through the G1 phase of the cell cycle, through phosphorylation of the retinoblastoma (Rb) protein.<sup>17</sup> Overexpression of cyclin D1 in rat fibroblasts enhances their growth and tumorigenicity,<sup>18</sup> and cyclin D1 has also been implicated as an oncogene in human cancers of diverse histogenesis.<sup>19–24</sup> Recently, cyclin D1 has been implicated as a putative proto-oncogene in transitional cell carcinomas (TCCs) of the urinary bladder, and its overexpression is correlated with tumor grade.<sup>25</sup> Cyclin D1 expression is inducible by wild-type p53 protein in the normal cell-cycle transition and this induction is at least partially mediated by p21<sup>WAF1/Cip1</sup> (also known as WAF1 and Cip1), a potent CDK inhibitor which blocks DNA replication.<sup>26, 27</sup> Mutations of the p21<sup>WAF1/Cip1</sup> gene have been reported in several kinds of human tumors and recently in urinary bladder carcinoma.<sup>28</sup> While alterations of p53, PCNA, cyclin D1 and p21<sup>WAF1/Cip1</sup> protein expression are often found in tumors, the situation in bladder urothelium of people with long-term exposure (more than 11 years) to low doses of ionizing radiation has remained unclear. However, our preceding pilot studies indicated increased proliferative activity and possible genetic instability, with overexpression of p53, PCNA and mdm2 in the bladder urothelium.<sup>4, 29</sup>

To evaluate further the role of p53 and differences in expression of PCNA, cyclin D1 and the CDK inhibitor p21<sup>WAF1/Cip1</sup> with particular reference to precancerous lesions, the present immunohistochemical (IHC) study of bladder urothelium of patients with benign prostatic hyperplasia (BPH) living in radiocontaminated areas of Ukraine was conducted.

## MATERIALS AND METHODS

**Urinary bladder samples** Formalin-fixed, paraffin-embedded tissue blocks from 55 male patients who underwent surgery at the Institute of Urology and Nephrology, Academy of Medical Sciences of Ukraine in Kiev, Ukraine, in 1995 and 1996 in connection with BPH were examined. Multiple mapping biopsies of the bladder urothelium were taken from every patient. A total of 228 paraffin-embedded specimens from 55 patients were immunohistochemically investigated.

Patients who lived for at least 10 years in the radiocontaminated areas of Ukraine, including Kiev city, after the Chernobyl accident were divided into two groups. Group I

consisted of 28 individuals (62 years old on average), inhabiting radiocontaminated areas of Ukraine with <sup>137</sup>Cs contamination densities from 5 to 30 Curies per square km. Group II consisted of 17 patients from Kiev city (75 years old on average) with a density of <sup>137</sup>Cs contamination from 0.5 to 5 Curies per square km. The measurement of one-day urine radioactivity of patients from groups I and II was performed using an ADCAM-100 instrument with the GEM-50250 germanium detector (ORTEK, USA). The level of <sup>137</sup>Cs in one-day urine in patients of group I was 0.8 to 28.9 Bq/liter and that of group II was 0.22 to 8.1 Bq/liter. The group III controls included 10 patients (66 years old on average) living in so-called "clean" areas of the country (without radiocontamination, but with possible chemical contamination, as Ukraine is considered an ecological disaster area). The patients of all three groups had similar nutritional status and the majority of them had smoked tobacco for more than 20 years (about 20 cigarettes a day).

**Histopathology and IHC staining** Four to 5  $\mu$ m sections of bladder samples were stained with hematoxylin and eosin (H & E). The bladder lesions were classified in accordance with the histological criteria defined by the World Health Organization's (WHO) International Classification of Tumors. Histological criteria for mild, moderate and severe dysplasias were used in the interpretation of flat noninvasive lesions of the urothelium.<sup>30</sup> IHC staining was performed using a standard avidin-biotin peroxidase complex (ABC) method with the Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA). Briefly, after deparaffinization of the tissue sections in xylene and dehydration through graded series of alcohols, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in distilled water for 5 min. The sections were rinsed in distilled water, and microwave treatment was performed in distilled water for 22 min at low power. For p53, PCNA and p21<sup>WAF1/Cip1</sup> IHC staining, sections were blocked with goat serum at 37°C for 30 min. Mouse monoclonal antibodies against p53 (DO-7 immunoglobulin IgG2b, DAKO, Glostrup, Denmark) at 1:100 dilution; PCNA (DAKO) at 1:500 dilution (PC-10, IgG2a, DAKO); and p21<sup>WAF1/Cip1</sup> (SC-187, mouse monoclonal Ab, Santa Cruz Biotechnology, Inc., CA) at 1:500 dilution were then applied as primary antibodies overnight at 4°C. For cyclin D1 IHC staining, sections were blocked with horse serum and then incubated with rabbit polyclonal antihuman cyclin D1 antibody (rabbit polyclonal IgG, UBI, Lake Placid, NY) at 1:200 dilution. Staining was achieved using a Vectastain ABC-PO kit and 3,3'-diaminobenzidine tetrahydrochloride (Wako, Tokyo) as the chromogen. As controls, known positive tissue sections were used and for negative controls, exposure to the primary antibody was omitted. The IHC analysis was performed in a blind fashion without knowledge of the patient group or any clinical

Table I. Incidence of Urinary Bladder Dysplasia and Carcinoma in the Different Groups

Group	No. of cases	Moderate to severe dysplasia (%)	Urinary bladder carcinomas		
			Total (%)	CIS	Papillary TCC with/without invasion
I	28	26 (93) <sup>b</sup>	15 (54) <sup>b</sup>	14	1
II	17	16 (94) <sup>b</sup>	9 (53) <sup>b</sup>	8 <sup>a</sup>	1
III	10	4 (40)	0	0	0

Significantly different from group III at: a)  $P < 0.05$ , b)  $P < 0.01$  (Fisher's exact probability test). CIS, carcinoma *in situ*; TCC, transitional cell carcinoma.

information. Consecutive serial sections were used for IHC and H & E staining. All specimens were evaluated independently by at least two pathologists; approximately 12–16 sections per case were analyzed.

**Quantitative analysis** IHC analysis was performed using a system for evaluation and grading of the immunostaining pattern (IP) with multiple values for extent and intensity to give scores of 0–9.<sup>31)</sup> The extent of the staining was scored on a semiquantitative scale of 0 to 3, using the following criteria: 0, no detectable staining; 1, less than 10% scattered stained nuclei; 2, more than 10% but less than 50% stained nuclei; 3, homogeneous staining in more than 50% of the cell layers. The percentage of cells expressing p53, PCNA, cyclin D1 and p21<sup>WAF1/Cip1</sup> was determined by counting 500 cells per slide. The intensity of the staining was scored using the following criteria: 0, no detectable staining; 1, weakly stained nuclei; 2, moderately stained nuclei; 3, strongly stained nuclei. The final scores were derived from multiplication of extent by intensity. For example, urothelium with a score of 9 had an extent of IP > 50% and the strongest intensity (3 × 3 = 9).

**Statistical analysis** Statistical analyses were performed using Stat View software (Abacus Concepts Inc., Berkeley, CA). Fisher's exact probability test was used for incidences of lesions and the Mann-Whitney's *U*-test was used to examine differences when *P* values were less than 0.05.

## RESULTS

**Histopathology** In groups I and II, multiple regions of moderate to severe dysplasia in the urinary bladder were detected in 93% and 94% of patients, respectively (Table I). About half of the cases demonstrated areas of carcinoma *in situ* (CIS), this being significant as compared to group III. Two small TCC (one papillary and one invasive form, stage 1) were found in one patient of each of groups I and II. There were no significant differences in urinary bladder lesions between groups I and II. All cases of groups I and II exhibited proliferative cystitis, i.e. von Brunn's nests, cystitis cystica, squamous and glandular metaplasias, which were frequently combined (no signifi-

cant difference from group III, Table II). Umbrella cells of bladder urothelium, which partially remained in 50% of the specimens, showed degenerative cytoplasmic changes. Dilated blood vessels, hemorrhage, and angiomatoid vascular changes, as well as areas of sclerosis, were apparent in bladder submucosa of most patients. They showed the features of "radiation cystitis" rather than simple inflammation.

In the control group (group III), the umbrella cells were generally intact without degenerative changes in 80% of the cases. Areas of mild dysplasia were seen in 4 (40%) cases (Table I).

**Immunohistopathology** Patterns of IHC staining of areas of dysplasia, CIS, small papillary TCC and squamous metaplasia are illustrated in Figs. 1 and 2. The results for IHC status (p53, PCNA, cyclin D1, and p21<sup>WAF1/Cip1</sup> expression) of the bladder urothelium in groups I–III are shown in Figs. 3–5.

**p53 expression** Analysis of 140 paraffin sections from 45 patients with BPH in groups I and II demonstrated positive nuclear staining for p53 in 27 (60%) cases, the extent correlating with the intensity. A good correlation on the semiquantitative scale for p53 extent and intensity was observed among the three groups (Fig. 3). Group I had the strongest staining followed in order by groups II and III. Their scores are shown in Fig 4a. For example, in group I, 3 (10%) of 28 urothelia examined were scored as 9 and 1 (3.6%) was scored as 6. The average scores for p53 were 2.3 in group I, 0.8 in group II and 0.1 in group III (Fig. 4a). Many umbrella cells had nuclei positive for p53. The strongest nuclear overexpression of p53 was seen in the basal and intermediate layers. Areas of moderate to severe dysplasia and CIS showed high scores of p53. In von Brunn's nests and areas of squamous metaplasia with dysplasia, strong p53 expression was present diffusely. Normal urothelium was scored as 0 or 1.

**PCNA expression** The majority of specimens from groups I and II showed increased PCNA expression. There was a good correlation between PCNA staining extent and staining intensity in all three groups (Fig. 3). Strong PCNA expression was seen not only in the basal

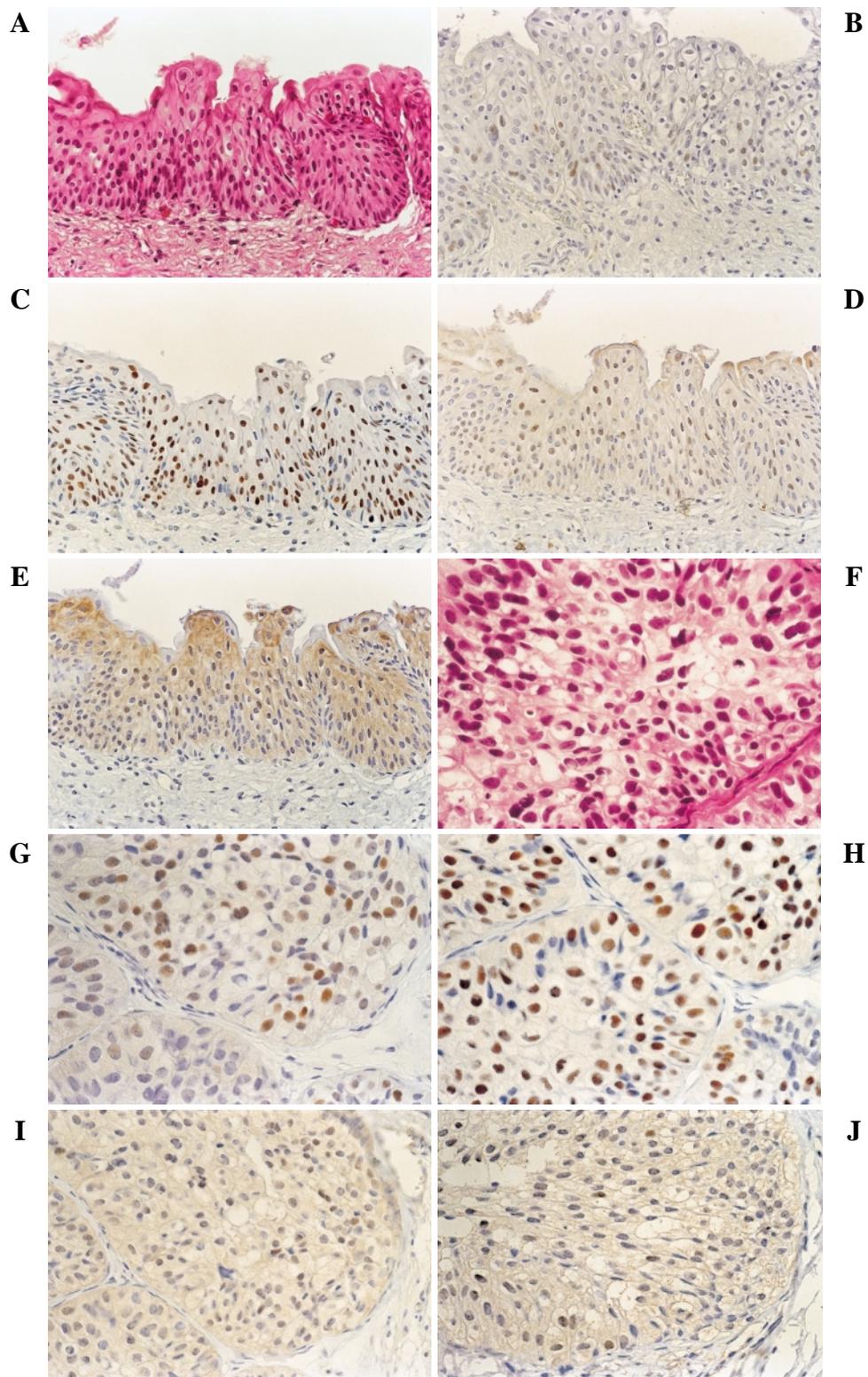


Fig. 1. IHC findings for bladder urothelium of a male exposed to long-term, low-dose ionizing radiation. A–E, moderate dysplasia. A, H & E; B, p53 expression; C, PCNA expression; D, cyclin D1 expression; E, p21<sup>WAF1/Cip1</sup> expression. F and G, von Brunn's nests with CIS grade II. F, H & E; G, p53 expression; H, PCNA expression; I, cyclin D1 expression; J, p21<sup>WAF1/Cip1</sup> expression. (magnification: A–E, ×90; F–J, ×180)

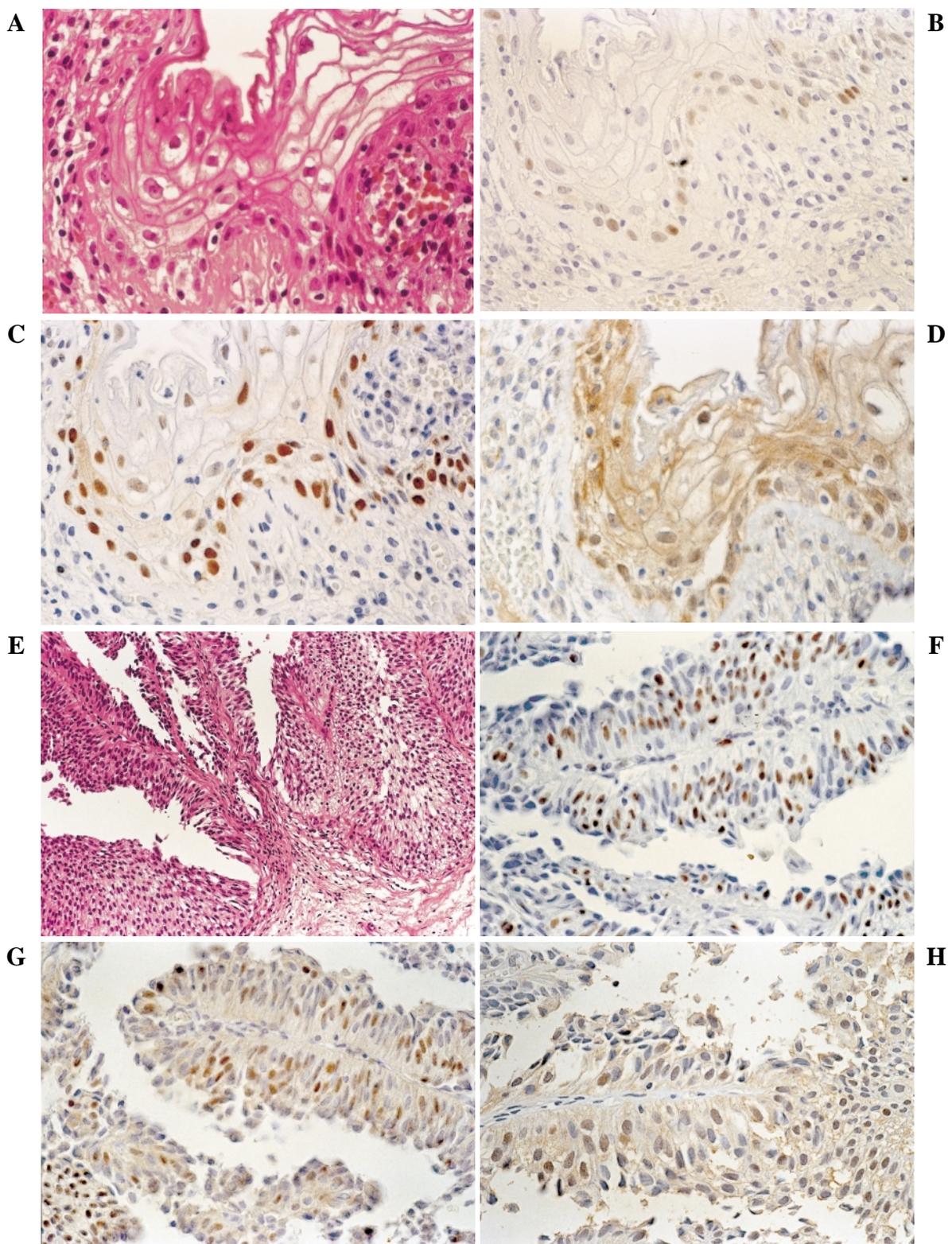


Fig. 2. IHC findings. A-D, squamous metaplasia of bladder urothelium. A, H & E; B, p53 expression; C, PCNA expression; D, cyclin D1 expression. E-H, TCC stage I. E, H & E; F, PCNA expression; G, cyclin D1 expression; H, p21<sup>WAF1/Cip1</sup> expression. (magnification: A-D and F-H,  $\times 230$ ; E,  $\times 35$ )

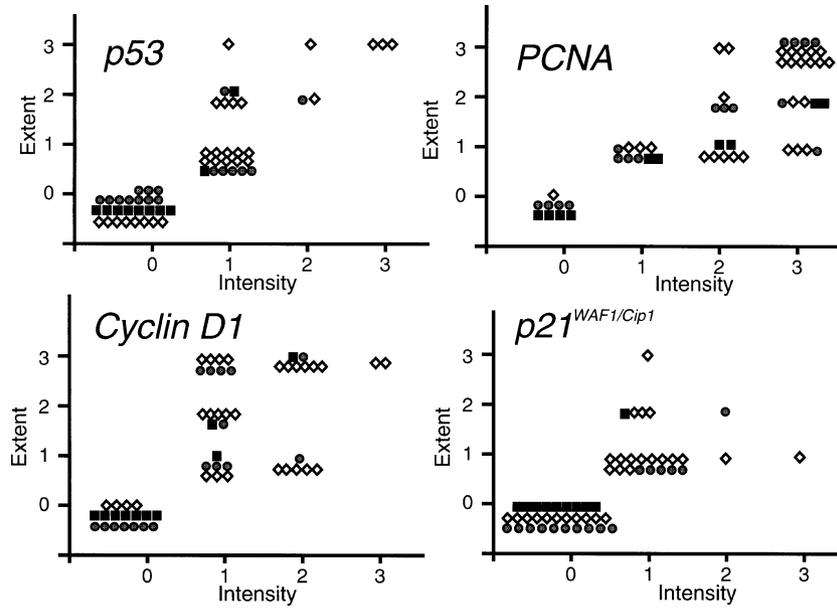


Fig. 3. Summary of IHC findings for bladder urothelium in groups I-III.  $\diamond$  group I ( $n=28$ ),  $\bullet$  group II ( $n=17$ ),  $\blacksquare$  group III ( $n=10$ ).

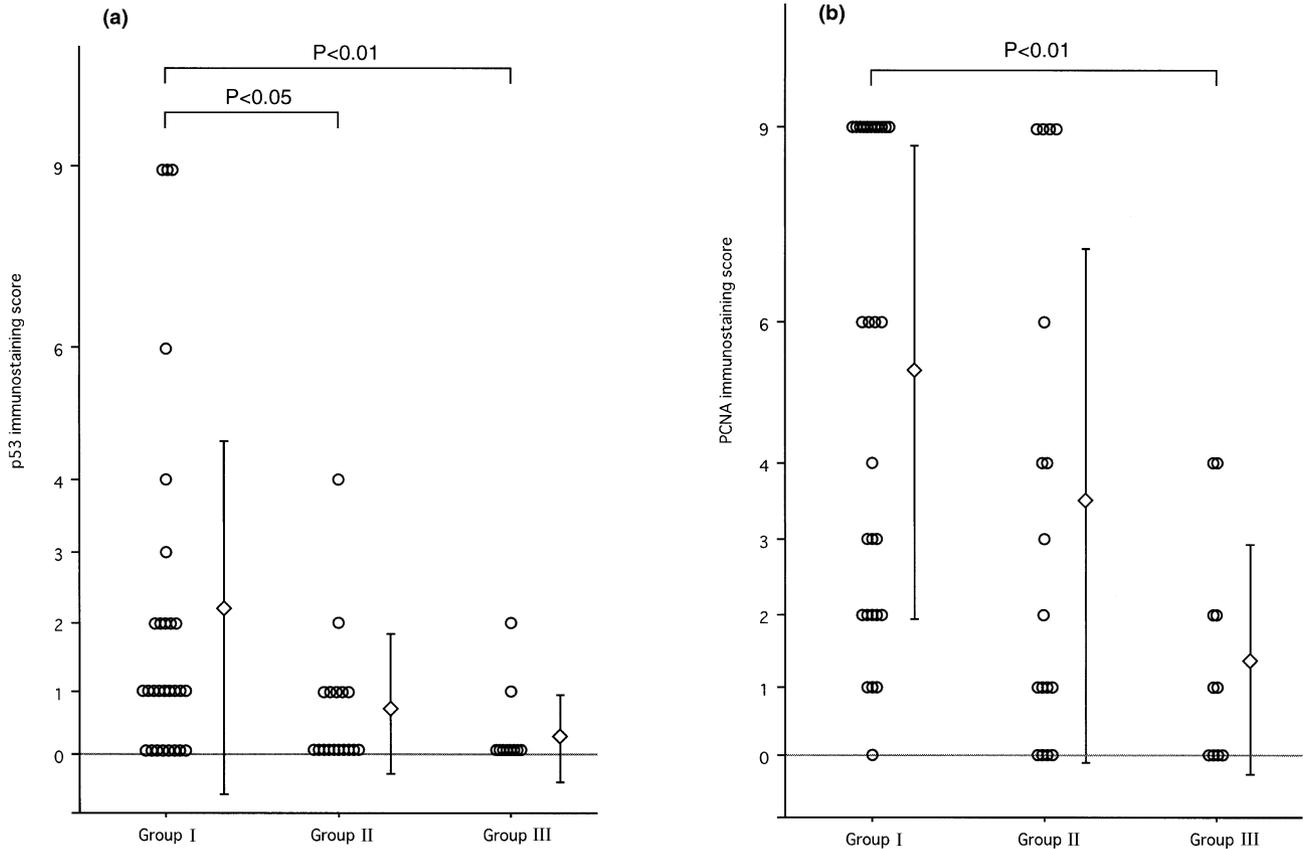


Fig. 4. IHC scores for bladder urothelium in groups I-III. (a) p53 scores. (b) PCNA scores. Vertical bars, mean  $\pm$  SD. Mann-Whitney's  $U$ -test was used for statistical analysis.

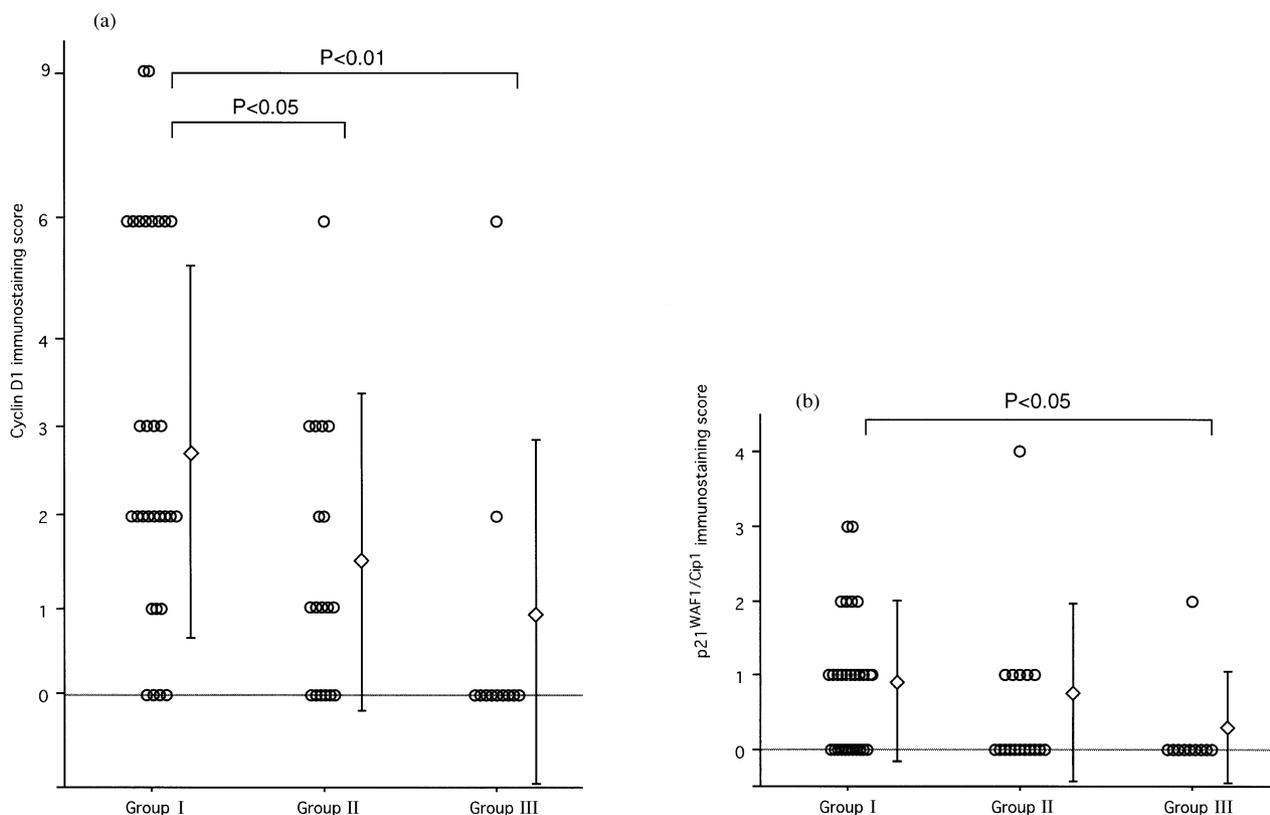


Fig. 5. IHC scores for bladder urothelium in groups I-III. (a) Cyclin D1 scores. (b) p21<sup>WAF1/Cip1</sup> scores. Vertical bars, mean ± SD. Mann-Whitney's *U*-test was used for statistical analysis.

Table II. Histological Changes in the Urinary Bladder Epithelium of the Different Groups

Group	No. of cases	Chronic non-proliferative cystitis	Follicular cystitis	von Brunn's nests	Cystitis cystica	Glandular metaplasia	Squamous metaplasia	Combined forms
I	28	3	1	3	1	1	6	13
II	17	5	0	3	1	0	1	7
III	10	3	1	2	0	0	1	3

Combined forms mean the combination of different histological forms.

cells, but also in the nuclei of intermediate and even superficial layers of the urothelium and in the nuclei of basal cells of squamous metaplasia. The average scores for PCNA staining were 5.5 for group I, 3.5 for group II and 1.4 for group III (Fig. 4b).

**Cyclin D1 expression** A good correlation between the extent and intensity of cyclin D1 staining was observed for all three groups (Fig. 3). Approximately 75% of specimens in groups I and II were stained positively. In cyclin D1-positive specimens, positive nuclear staining was evident in the nuclei of the whole layers of urothelium, von

Brunn's nests and squamous metaplasia, especially in areas of dysplasia. The areas of severe dysplasia and CIS were cyclin D1-positive and were scored as 6. In the control group III, 9 (90%) cases were cyclin D1-negative. The average scores were 2.8 in group I, 1.7 in group II and 1 in group III (Fig. 5a).

**p21<sup>WAF1/Cip1</sup> expression** Only a weak correlation between p21<sup>WAF1/Cip1</sup> extent and intensity was observed in the three groups (Fig. 3). The average scores for p21<sup>WAF1/Cip1</sup> expression were 1 in group I, 0.8 in group II and 0.2 in group III (Fig. 5b). p21<sup>WAF1/Cip1</sup> staining was most striking in the

nuclei of cells of the superficial layer of the urothelium, including umbrella cells. As for areas of dysplasia or CIS, nuclear staining of p21<sup>WAF1/Cip1</sup> was frequently observed in epithelial cells of the basal layer and intermediate compartments. Areas of squamous metaplasia were mostly p21<sup>WAF1/Cip1</sup>-negative.

## DISCUSSION

The present results provide compelling evidence of a dramatic increase in the incidences of moderate to severe dysplasia and CIS of the urinary bladder in male patients living in the radiocontaminated areas of Ukraine, and to a lesser extent in Kiev city, since the Chernobyl accident. Two small TCCs were incidentally found in these patients. In addition, correlations between p53, PCNA, cyclin D1 and p21<sup>WAF1/Cip1</sup> IHC findings and pathologic changes in bladder urothelium were observed in these patients. The staining intensities of p53, PCNA, p21<sup>WAF1/Cip1</sup> and cyclin D1 were directly correlated to levels of radiation exposure and the levels of <sup>137</sup>Cs excreted in the urine by patients of groups I and II. The patients from "clean" (not radio-contaminated) areas of Ukraine had significantly lower values for all parameters investigated. The rather high incidence of mild dysplasia (40%) in the control group may be explained by the fact that Ukraine is acknowledged to be an ecological disaster area. Other factors such as progressive chemical contamination of the environment may also lead to dysplastic changes of the urothelium.

It has been reported that ionizing radiation can induce cancer by activation of oncogenes such as *ras* and *myc*, or inactivation of tumor suppressor genes such as *p53* or *RB*.<sup>13,32</sup> Growth arrest after irradiation is thought to provide cells with additional time to repair DNA damage in late G1, G2 and possibly also S-phase.<sup>33</sup> The p53 tumor suppressor gene product is known to be a crucial target of ionizing radiation; its level dramatically increases in response to irradiation<sup>33</sup> and this leads to inhibition of DNA synthesis. Alternatively, defective p53 function could allow DNA replication to proceed with a damaged template, which may result in accumulation of genetic defects.<sup>34</sup> In our cases, radiation-induced DNA damage was presumably generated over several years.

Recently it has been shown that ultraviolet and ionizing radiation-induced lesions are repaired through two interrelated nucleotide excision repair pathways: transcription-coupled repair and bulk nucleotide excision repair.<sup>32</sup> It has been shown that wild-type p53 plays an essential role in transcription-coupled repair of DNA damage induced by oxidative stress, ionizing radiation and some chemical carcinogens.<sup>35</sup> The weak staining of p53-positive nuclei of bladder urothelium in both radiocontaminated groups likely represents wild-type p53 accumulation in response

to DNA damage. This speculation is supported by aberrant increases of PCNA, cyclin D1 and p21<sup>WAF1/Cip1</sup> in the same areas of urothelium, that could be explained by possible involvement of PCNA, cyclin D1 and p21<sup>WAF1/Cip1</sup> not only in DNA synthesis, but also in DNA excision repair.<sup>14,36</sup> Activation of cyclin D1 and p21<sup>WAF1/Cip1</sup> occurs in response to activation of wild-type p53. Furthermore, cyclin D1 has been found to accumulate in the nuclei of G1 phase cells and to disappear when the cells enter S-phase.<sup>36</sup> Recent studies have shown that p53 has at least two functions in relation to DNA repair: one is check point control, through transcriptional activation of p21<sup>WAF1/Cip1</sup> and the other is as an inducer of apoptosis.

It can be speculated that long-term exposure to low doses of <sup>137</sup>Cs induces chronic DNA damage in bladder urothelium. An alternative explanation for the strong p53 immunoreactivity observed in CIS and TCC as well as in some regions of dysplasia is that it reflects mutations within the *p53* gene. Using DNAs extracted from semi-serial paraffin-embedded sections, we are currently embarking on a polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis for *p53* gene mutations.

Elevated p21<sup>WAF1/Cip1</sup> protein levels accompany prolonged radiation-induced G1 arrest in fibroblasts.<sup>37</sup> Recent studies have shown that radiation-induced arrest is reversible.<sup>38</sup> The increased level of p21<sup>WAF1/Cip1</sup> could thus be interpreted as a manifestation of cellular differentiation. p21<sup>WAF1/Cip1</sup> directly interacts with PCNA but the majority of studies show that it does not inhibit PCNA-dependent nucleotide excision repair.<sup>14</sup> This suggests that p21<sup>WAF1/Cip1</sup> not only plays a critical role in negatively controlling proliferation, but also could be activated in a p53-independent manner.<sup>39</sup>

About 75% of our cases in both groups I and II were cyclin D1-positive. It is possible that some of the observed cyclin D1 overexpression in our samples occurred in response to activation of wild-type p53 during radiation-induced DNA damage. Besides, it is of interest to note that the overexpression of cyclin D1 in rats is also found in early precancerous lesions of the bladder urothelium induced by the chemical carcinogen, N-butyl-N-(4-hydroxybutyl)nitrosamine.<sup>40</sup>

The dramatic increase of PCNA staining in 88% of cases in groups I and II involving not only the basal cells, but also the suprabasal and superficial layers of bladder urothelium is indicative of increased cell proliferation. However, recent studies have shown that this nuclear factor also plays an essential role in nucleic acid metabolism as a component of the replication and repair machinery.<sup>16</sup>

In conclusion, the present finding of significant increases in p53, PCNA, cyclin D1 and to a lesser extent p21<sup>WAF1/Cip1</sup> expression in the majority of patients in groups I and II chronically exposed to ionizing irradiation,

along with multiple areas of cellular atypia, dysplasia, CIS and even 2 cases with small TCC, is suggestive of an increased chance of malignant transformation with the possible development of bladder cancer among the population in Ukraine. These data emphasize the necessity of long-term follow-up of people living in the radiocontaminated areas of Ukraine, preferably by multiparameter assessment including regular cystoscopy and cytological analysis of cells obtained by bladder washing. A prospective study of younger populations would also be worthwhile.

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