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ORIGINAL ARTICLE

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Radiation induces *p53*-dependent cell apoptosis in bladder cancer cells with wild-type-*p53* but not in *p53*-mutated bladder cancer cells

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Abstract Purpose. It has been reported in several studies that the absence in cancer cells of the *p53* tumor suppressor gene, mutations of which are frequently found in bladder cancer, increases their resistance to ionizing radiation. Other studies, however, suggest that mutations of the *p53* gene could increase the radiosensitivity of cancer cells, although the evidence is still inconclusive. In the present study, we investigated the relationship between *p53* status and radiation response in five different bladder cancer cell lines. Materials and Methods. Five different human bladder cancer cell lines (KK47: with wt-*p53*, RT4: with wt-*p53*, T24: with mutated *p53*, 5637: with mutated *p53*, UM-UC-3: with mutated *p53*) were used in the study. Cells were irradiated with 0, 2, 4, 6 or 8 Gy, then trypsinized and re-plated for clonogenic survival assay, quantitative RT-PCR assay, flow-cytometry analysis and TUNEL assay. Results. The clonogenic assay demonstrated that KK47 and RT4 had significantly higher radiosensitivity than other cell lines. Quantitative RT-PCR analysis showed that radiation induced increased expression of *p53*, *Bax*, and *p21* mRNA in KK47 and RT4. After irradiation, G1 cell-cycle arrest was observed in KK47 and RT4 under flow cytometry analysis, while T24, 5637, and UM-UC-3 showed an increase in the proportion of G2 cells. Increased cell apoptosis was also observed under TUNEL assay in KK47 and RT4, but not in other cell lines. Conclusions: It was demonstrated that ionizing radiation induces *p53*-dependent cell apoptosis in bladder cancer cells with wt-*p53* but not in those with mutated *p53*.

Keywords Bladder cancer · *p53* · Radiation

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Introduction

Bladder cancer is the second most common genitourinary malignancy, with transitional cell carcinoma (TCC) comprising nearly 90% of all primary bladder tumors. Each year in the United States nearly 55,000 patients are diagnosed and 12,000 die from the disease [1]. Although the majority of patients present with superficial bladder tumors, 20–40% either present with or develop invasive disease. Bladder cancer in the form of TCC has been recognized as moderately radiosensitive and radiotherapy has recently been used frequently for the treatment of locally advanced disease in combination with transurethral tumor resection (TUR) and/or chemotherapy as part of a multimodal bladder preservation therapy [2].

Current ideas suggest that the *p53* tumor suppressor gene, mutations in which are frequently found at in bladder cancer [1, 3], functions as a central control checkpoint in response to the DNA damage induced by a variety of agents, including ionizing radiation (IR), and plays an important role in the induction of apoptosis [4, 5, 6, 7]. Cells with loss of *p53* function induced by loss or mutation of the *p53* gene may

therefore show increased resistance to IR due to the loss of growth-arrest and/or apoptosis mechanisms.

Although there are many published reports indicating that *p53* is an important determinant of radiosensitivity [6, 7, 8, 9, 10, 11], controversy continues over this relationship, and some studies have reported that mutations in the *p53* gene can lead to an increase in radiosensitivity, possibly due to the loss of *p53*-dependent DNA repair [12, 13], yet other reports suggest that loss of *p53* has no effect on radiosensitivity [14, 15]. There is also some evidence to suggest that any correlation between *p53* function and radiosensitivity may be a tissue-specific phenomenon [15]. Thus the relationship between *p53* status and radiosensitivity remains unclear.

Since radiotherapy is frequently used to treat bladder cancer and this cancer has a high incidence of *p53* mutation [16], it is important to investigate the possible relationship between *p53* status and radiosensitivity in bladder cancer cells. In the present study, we examined the possible role of *p53* in the response to IR of three different human bladder cancer cell lines: two expressing wt-*p53* and the others containing mutations of the *p53* gene. Our data suggest that the cell lines expressing wt-*p53* were more sensitive to IR than the other three containing mutations of the *p53* gene, possibly due to *p53*-dependent cell apoptosis.

Materials and methods

Cells and culture conditions

The established cell lines from TCC human bladder cancer, 5637, T24, and RT4 were obtained from the American Type Culture Collection (Rockville, MD). UM-UC-3 was obtained from Dainippon Pharmaceutical Co. (Osaka, Japan). KK47 was generously provided by Dr. Seiji Naito (Dept. of Urology, Kyushu University, Fukuoka, Japan). The cells were maintained in RPMI-1640 (GIBCO, MD) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin (Sigma Chemical Co., St. Louis, MO) at 37°C and 5% $C0_2$. The 5637 cells display two mutations, at codon 280 (Arg > Thr) and codon 72 (Arg > Pro) [17, 18], UM-UC-3 cells display two mutations, at codon 113 (Phe → Cys) and codon 72 (Arg → Pro) [17, 18], while T24 cells contain a *p53* allele encoding an in-frame deletion of tyrosine 126 [18], no mutation is detected in KK47 and RT4 Table 1) [17, 18, 19, 20].

Irradiation

Cell cultures were irradiated at room temperature in tissue culture flasks with 0–8 Gy at a dose rate of approximately 300 cGy/min using a Mitsubishi linear accelerator. The cells were then trypsinized and plated for clonogenic assay, quantitative RT-PCR, TUNEL assay, and flow-cytometry analysis.

Clonogenic survival assay

For determination of clonogenic ability following irradiation, cells were plated in p-100 dishes at a density of 1000 cells/well. After 9-day incubation at 37°C, colonies were stained with crystal violet in 50% methanol and the number of colonies of 20 or more cells was counted. Surviving fraction values were then fitted to a single-hit multitarget (SHMT) model: S/S0 = 1-(1-e-D/D0)n, where S/S0 is the surviving fraction and D the dose (Gy). The parameters n and D0 were calculated using Kaleida Graph software (Synergy Software, Reading, PA).

Extraction of total RNA

To examine the effects of IR on the expression of messenger RNA (mRNA) by *p53*, *Bax* and *p21* in five cell lines, the cells were re-plated onto 100-mm culture plates after 4 Gy of irradiation. Two days after re-plating, the cells were trypsinized and the cell pellets were collected by centrifugation at 1000 *g* for 5 min. Isogen (Nippon Gene, Tokyo, Japan) was used to extract the total RNA from the cells.

Quantitative RT-PCR using TaqMan fluorogenic detection system

Quantification of *p53*, *Bax* and *p21* mRNA expression with quantitative RT-PCR was performed using the TaqMan fluorogenic detection system as previously described [21]. Briefly, primers and the TaqMan probe for *p53*, *Bax* and *p21* were designed using the primer design software Primer Express (Perkin-Elmer Applied Biosystems, Foster City, CA). Table 2 shows the sequences for the TaqMan probes and primers used. Primers and the TaqMan probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; TaqMan GAPDH control reagent kit) were purchased from Perkin-Elmer Applied Biosystems. RNA for GAPDH was used as an endogenous control. Fifty microliters of reaction mixture were used, containing 10 ng of the extracted total RNA; 1x TaqMan buffer A; 5.5 mmol/l MgCl2, 300 μmol/l dATP, dGTP and dCTP; 600 μmol/l dUTP; 0.2 μmol/l forward and reverse primers; 0.1 μmol/l TaqMan probe; 1.25 U of AmpliTaq Gold; 12.5 U of MuLV reverse transcriptase; and 20 U of RNase Inhibitor (Perkin-Elmer Applied Biosystems). The conditions of one-step RT-PCR were as follows: 30 min at 48°C (stage 1, RT), 10 min at 95°C (stage 2, RT inactivation and

Table 1 *p53* gene mutations in bladder cancer cells

Cell line	No. of 17p alleles	Codon	Nucleotide change	Amino acid change
5637	2	280	AGA → ACA	Arg → Thr
UM-UC-3	2	72	CGC → CCC	Arg → Pro
		113	TTC → TGC	Phe → Cys
		72	CGC → CCC	Arg → Pro
T24	1	126	In-frame del TAC	del Tyr
KK47		No mutations detected in exons 4–9		
RT4		No mutations detected in exons 4–9		

Table 2 Primers and probes

p53	
p53 primer, forward;	5′-TGCGTGTGGAGTATTTGGATG-3′
p53 primer, reverse;	5′-TGGTACAGTCAGAGCCAACCAG-3′
p53 probe;	5′-AAACACTTTTCGACATAGTGTGGTGGTGCC-3′
Bax	
Bax primer, forward;	5′-AAGCTGAGCGAGTGTCTCAAGC-3′
p53 primer, reverse;	5′-TGGTACAGTCAGAGCCAACCAG-3′
Bax probe;	5′-CTGGACAGTAACATGGAGCTGCAGAGGA-3′
p21	
p21 primer, forward;	5′-AGCAGAGGAAGACCATGTGGAC-3′
p21 primer, reverse;	5′-TTTCGACCCTGAGAGTCTCCAG-3′
p21 probe;	5′-TGTCACTGTCTTGTACCCTTGTGCCTCG-3′
GAPDH	
GAPDH primer, reverse;	5′-GAAGATGGTGATGGGATTTC-3′
GAPDH primer, reverse;	5′-GAAGATGGTGATGGGATTTC-3′
GAPDH probe;	5′-CAAGCTTCCCGTTCTCAGCC-3′

AmpliTaq Gold activation), and then 40 cycles of amplification for 15 s at 95°C and 1 min at 60°C (stage 3, PCR). The assay used an instrument capable of measuring fluorescence in real time (ABI prism 7700 Sequence Detector, Perkin-Elmer Applied Biosystems). The data of the assay was analyzed using the analyzing software Sequence Detector (Perkin-Elmer Applied Biosystems).

Cell-cycle analysis with FACScan

To analyze the effect on cell-cycle checkpoint, five cell lines were re-plated onto 100-mm culture plates after a 4-Gy dose of irradiation. Two days after re-plating, adherent cells were harvested and fixed on ice for 30 min in PBS (pH 7.4) containing 2% formaldehyde. After several washings, cells were centrifuged and resuspended in 3 ml 80% ice-cold ethanol for post-fixation, and stored at –20°C. Prior to flow cytometry, cells were washed and incubated for 15 min in phosphate citric acid buffer (20% Triton X, 5 mg/ml RNase A in PBS) and resuspended in 50 mg/ml of propidium iodide. The cells were incubated for at least 15 min at room temperature in the dark, and DNA content of the preparations was analyzed with flow cytometry using a FACScan (Becton Dickinson, Franklin Lakes, NJ). A 488-nm laser was run at 15 mW, and

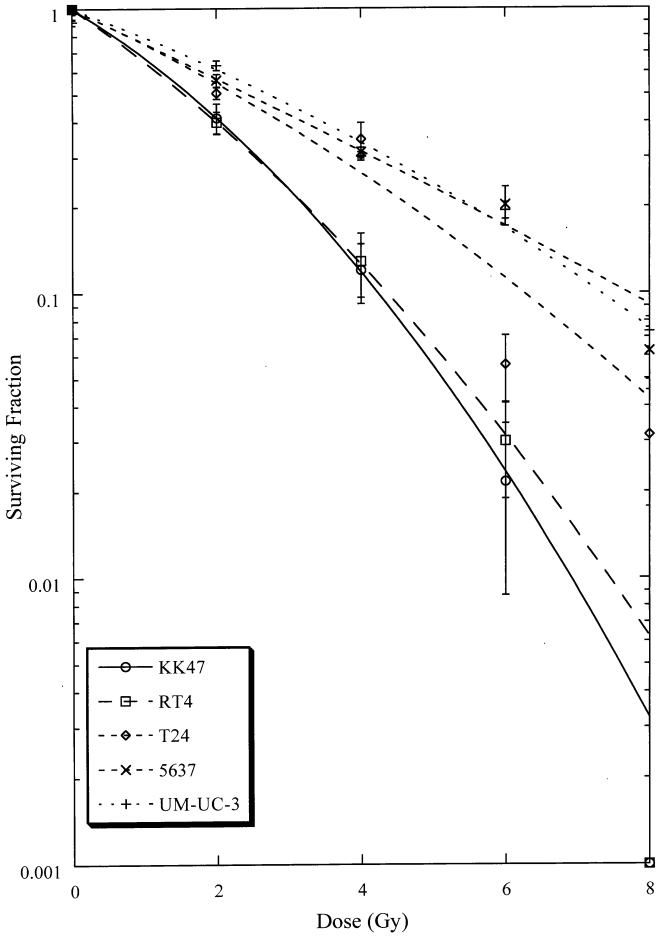


Fig. 1 Clonogenic survival profiles of the five human bladder cancer cell lines after exposure to graded doses of IR. Data are plotted as the mean and SE of three independent experiments (three dishes per point per experiment)

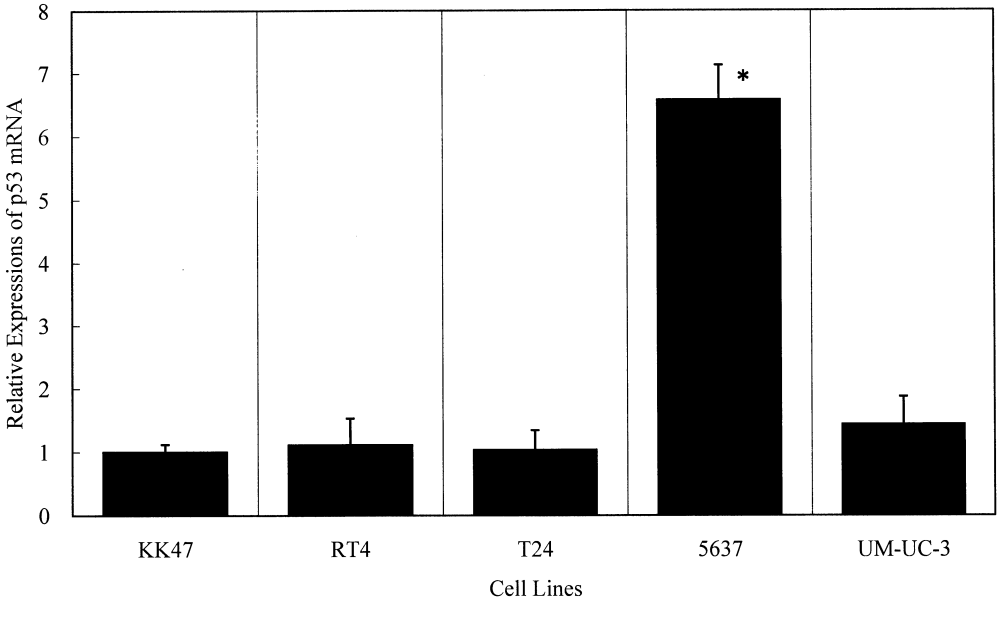


Fig. 2 Mean relative quantification of *p53* mRNA expression in five different cell lines. Values represent relative levels of *p53* mRNA expression normalized to the value of KK47 with triplicate determinations of each sample. GAPDH was selected as an endogenous RNA control to normalize for differences in the amount of total RNA. *p53* mRNA expression in 5637 cells was significantly higher than in the other cells ($*p < 0.05$)

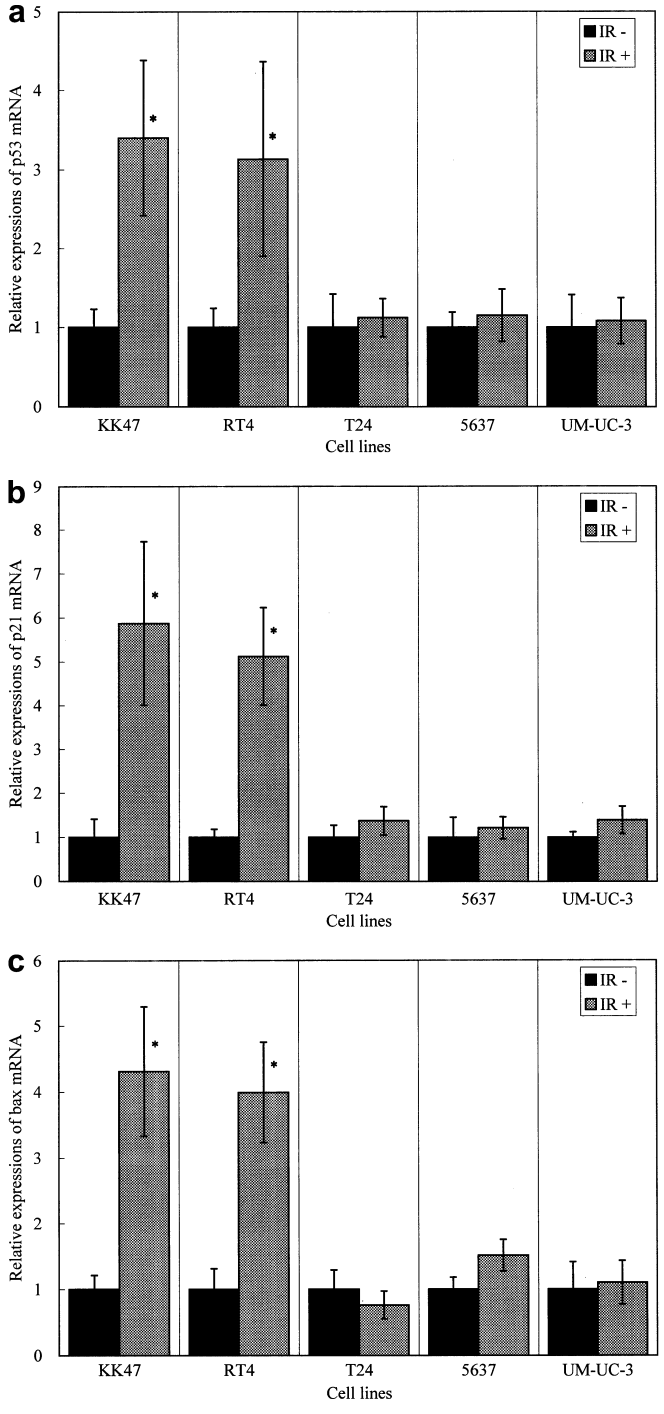


Fig. 3a–c Relative mRNA levels of *p53* (**a**), *Bax* (**b**) and *p21* (**c**) measured by quantitative RT-PCR of five human bladder cancer cell lines after exposure to 4 Gy of IR. Values represent relative levels of mRNA normalized to the values of corresponding nonirradiated cells with triplicate determinations of each sample. GAPDH was selected as an endogenous RNA control to normalize for differences in the amount of total RNA. IR induced a significant increase in *p53* mRNA in KK47 and RT4 cells ($p < 0.05$) but not in T24, 5637, or UM-UC-3 cells (**a**). IR thus induced a significant increase in *p21* and *Bax* mRNA expression only in KK47 and RT4 cells (**b**, **c**) (*$p < 0.05$)

Fig. 4a–j Representative DNA histograms of five human bladder cancer cell lines, stained with propidium iodide, using FACscan. **A** KK47 cells. **B** KK47 cells after IR. **C** RT4 cells. **D** RT4 cells after IR. **E** T24 cells. **F** T24 cells after IR **G** 5637 cells. **H** 5637 cells after IR. **I** UM-UC-3 cells. **J** UM-UC-3 cells after IR. The *arrows** represent G0/1 phase, and the *arrows*** represent G2/M phase

fluorescence was passed through a 585/42-nm band pass filter. At least 20,000 events were acquired using CELL Quest software (Becton Dickinson). The cell-cycle distribution was calculated using Mod Fit LT software (Becton Dickinson). Each experiment was performed at least twice.

Detection of apoptotic cells by TUNEL assay

Cells were evaluated for occurrence of apoptosis at 12 h and 2 days after IR (4 Gy). Apoptosis was assessed by morphological criteria and by an immunohistochemical process using the ApopTag assay kit (Oncor, Inc. Gaithersburg, MD, USA). This method detects nucleosome-sized DNA fragments by tailing the 3′-OH ends of the fragments with digoxigenin-nucleotide using terminal deoxynucleotidyl transferase [22]. DNA fragments were then tailed with digoxigenin-nucleotide using terminal deoxynucleotidyl transferase and incubated with an anti-digoxigenin antibody conjugated with diaminobenzidine substrate. Diaminobenzidine signals (brown) in the ApopTag assay and counter-staining with methyl green (green) were detected using a microscope. The percentage of TUNEL-positive cells was evaluated with a method for calculating apoptotic index (TUNEL-positive cells/all visible cells in each field.) The apoptotic index used was the average of five individual apoptotic indices.

Statistical analysis

Mann-Whitney's U test was employed for statistical analysis, and $p < 0.05$ was considered to be statistically significant.

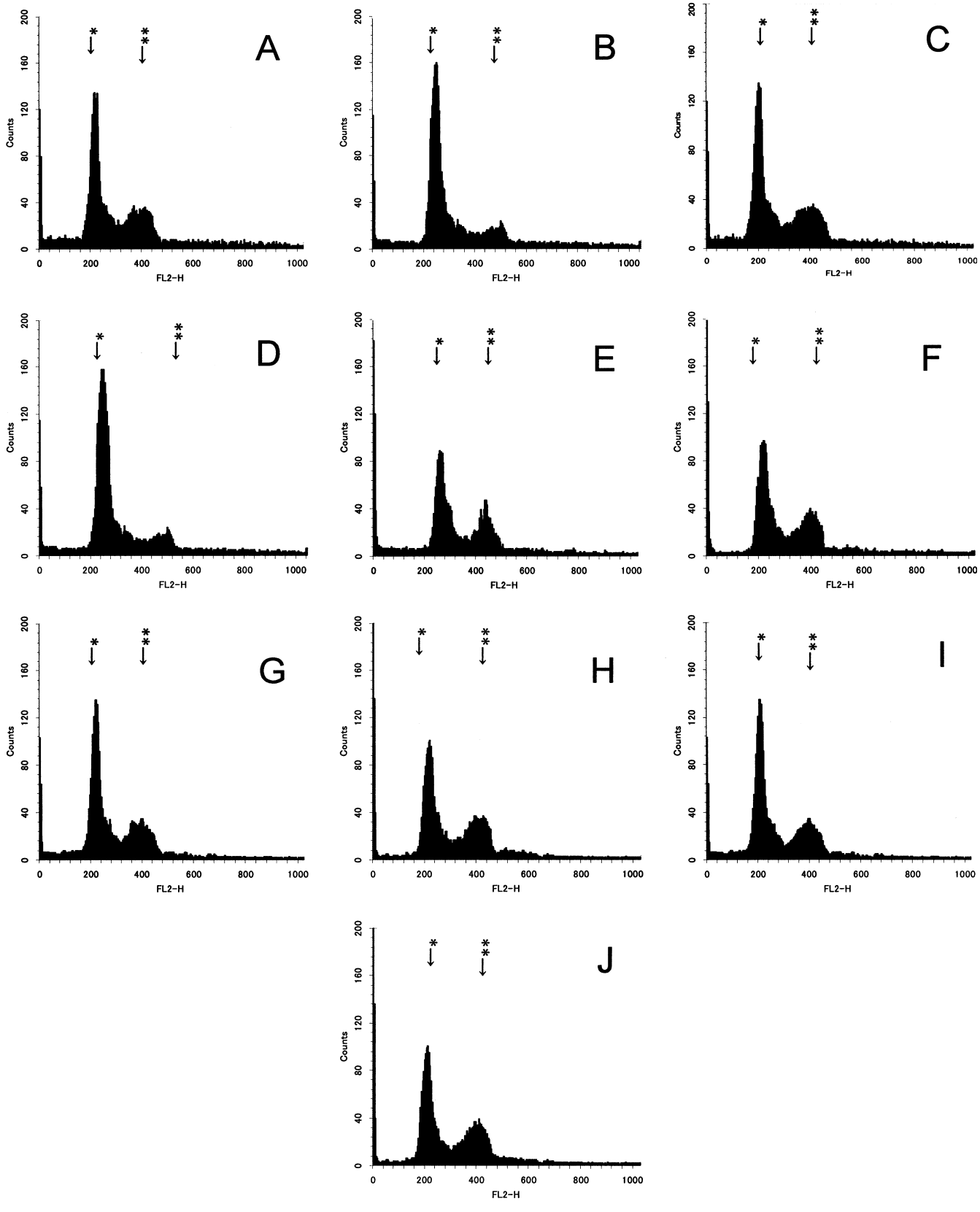
Results

Clonogenic survival assay demonstrated higher radiosensitivity in KK47 and RT4 cells than in T24, 5637 and UM-UC-3 cells

Clonogenic survival assay revealed significant and dose-dependent cell death in all five cell lines (Fig. 1). The 5637 and UM-UC-3 cells were found to have the highest level of clonogenic survival following IR treatment, while T24 cells were intermediately sensitive and KK47 and RT4 cells the most sensitive to IR.

The 5637 overexpresses *p53* mRNA

Figure 2 shows the mean relative quantification of *p53* mRNA expression in the five different cell lines. To normalize for differences in the amount of total RNA, GAPDH was selected as an endogenous RNA control. Relative quantification was performed by dividing the value for KK47. *p53* mRNA expression in 5637 cells was significantly higher than in the other cell lines ($p < 0.05$). A large number of studies have indicated that nuclear accumulation of *p53* shows a high correlation with mutations in the *p53* gene [1]. Of the five cell lines, KK47



and RT4 express wt-*p53*, T24 contains in-frame deletion in codon 126, 5637 contains point mutations in codons 72 and 280 and UM-UC-3 contains point mutation in codons 72 and 113. Overexpression of *p53* mRNA in 5637 cells may correlate with mutations of the *p53* gene in 5637.

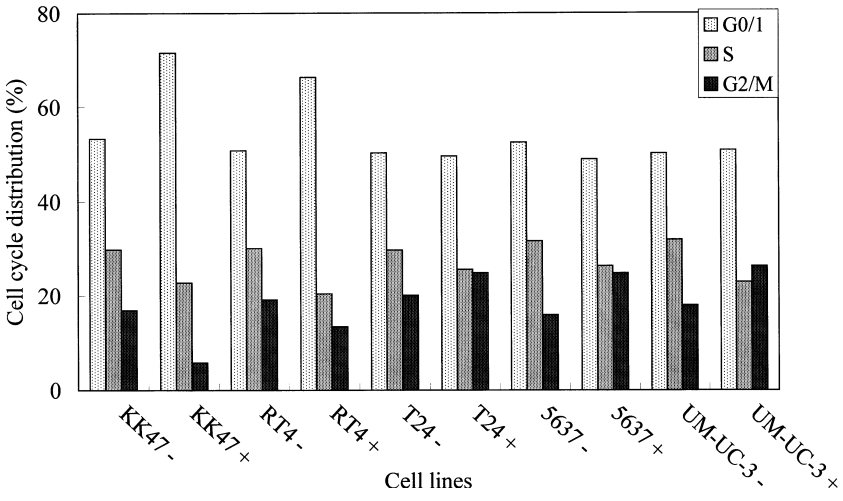


Fig. 5 Effect of irradiation on cell-cycle distribution (–, nonirradiated cells; +, irradiated cells). Following IR, G1 arrest was observed in the KK47 and RT4 cell lines and G2 arrest in the T24, 5637, and UM-UC-3 cell lines

IR induced significant increase in p53, p21 and Bax mRNA in KK47 and RT4 cells but not in T24, 5637, or UM-UC-3 cells

Figure 3a–c shows mean relative quantifications of *p53*, *p21* and *Bax* mRNA expression. To normalize for differences in the amount of total RNA, GAPDH was selected as an endogenous RNA control. Relative values in the irradiated cells were calculated by dividing the values for corresponding nonirradiated cells. IR induced a significant increase in *p53* mRNA in KK47 and RT4 cells ($p < 0.05$) but not in T24, 5637 and UM-UC-3 cells (Fig. 3a). IR subsequently induced significant increases in *p21* and *Bax* mRNA expression only in KK47 and RT4 cells (Fig. 3b, c) ($p < 0.05$).

IR induced G1 cell-cycle arrest in the KK47 and RT4 cell line, while G2 arrest was observed in the T24, 5637 and UM-UC-3 cell lines following IR

The effect on cell-cycle distribution was examined by flow cytometry 48 h after a 4-Gy dose of irradiation (Fig. 4). After IR treatment, G1 cell-cycle arrest was observed in KK47 and RT4, with an 18.1% and 15.4% increase in the proportion of G1 cells. In contrast, T24, 5637 and UM-UC-3 demonstrated respective 4.8%, 8.9%, and 8.3% increases in the proportion of G2 cells following IR (Fig. 5). This G2 arrest may represent a *p53*-independent mechanism, as neither cell line demonstrated elevation in *p53* gene expression after irradiation (shown in Fig. 3a).

IR induced cell apoptosis in KK47 and RT4 cells

IR induced cell apoptosis, as determined by TUNEL assay, in KK47 and RT4 cells (apoptotic index; $24.47 \pm 5.56\%$ and $20.31 \pm 4.21\%$) but not in T24 ($2.13 \pm 1.55\%$), 5637 ($0.25 \pm 0.21\%$), or UM-UC-3 ($1.27 \pm 0.24\%$) cells (Fig. 6, Table 3). The results showed a discrepancy between cell survival (Fig. 1) and frequency of cell apoptosis, which may result from different cell-death mechanisms (e.g., mitotic cell death) in response to IR treatment.

Discussion

Mutation in the *p53* gene, reported at frequencies of between 6% and 61% [3, 23, 24], is one of the most common genetic abnormalities found in bladder cancer. *p53* expression as determined by immunohistochemical analysis has been extensively evaluated as a prognostic factor for bladder cancer progression and response to chemotherapy and radiation therapy [25]. Wu et al. [26] found that of patients with extravesical disease who underwent preoperative radiotherapy, those with altered *p53* were at increased risk of distant metastasis and had a shorter survival time than those with wild-type *p53* expression. Osen et al. [27] found on the other hand that *p53* did not correlate with stage, grade, or outcome in patients with muscle-invasive TCC treated with definitive radiotherapy. The role of *p53* in the radiation-therapy response and survival of bladder cancer patients is thus controversial.

There are many studies indicating that *p53* plays an important role in a number of cellular processes such as cell-cycle regulation and induction of cell apoptosis in response to DNA damage including damage by IR [4, 5, 6, 7]. The relationship between *p53* status and radiosensitivity has been frequently reported, but remains the subject of controversy. Some studies suggest that absence of *p53* increases resistance to ionizing radiation due to the loss of growth arrest and/or apoptosis mechanisms [6, 8, 9, 10, 11], while other studies suggest that mutations in the *p53* gene sensitize to IR, possibly due to loss of *p53*-dependent DNA repair [12, 13].

In the present study, five different human bladder cancer cell lines, the KK47 and RT4 cell lines expressing

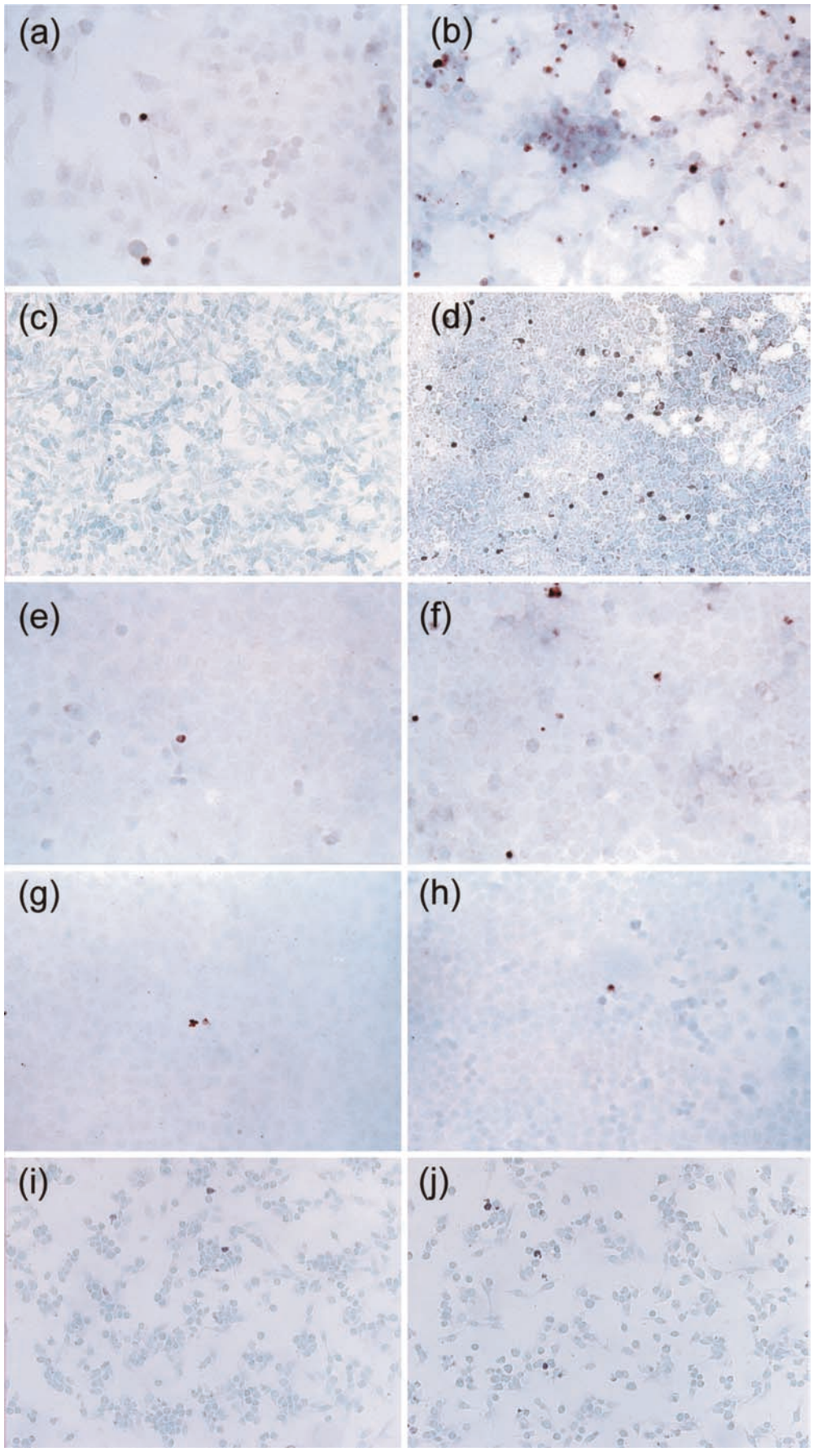


Fig. 6a–j Apoptotic cell detection by TUNEL assay was performed. **a** KK47 cells. **b** KK47 cells after IR. **c** RT4 cells. **d** RT4 cells after IR **e** T24 cells. **f** T24 cells after IR **g** 5637 cells. **h** 5637 cells after IR. **i** UM-UC-3 cells. **j** UM-UC-3 cells after IR Brown signals represent apoptotic cells. Green signals show nuclei counterstained with methylgreen. After IR, the ratios of brown cells were significantly higher in the KK47 and RT4 cell line than in the other cell lines

Table 3 Apoptotic Indices of KK47, RT4, T24, 5637 and UM-UC-3 cells (% ± SD)

	KK47	RT4	T24	5637	UM-UC-3
Non-irradiated cells	1.49 ± 0.63	1.21 ± 0.93	0.43 ± 0.35	0.12 ± 0.10	0.61 ± 0.18
Irradiated cells	24.47 ± 5.56	20.31 ± 4.21	2.13 ± 1.55	0.25 ± 0.21	1.27 ± 0.24

wt-*p53* and the T24, 5637, and UM-UC-3 cell lines containing mutations of the *p53* gene, were used to investigate the relationship between *p53* status and radiosensitivity in bladder cancer.

KK47 and RT4 contain a wild-type *p53* gene, which seems to be normally expressed. T24 cells contain a *p53* allele encoding an in-frame deletion of tyrosine 126. UM-UC-3 contains a mutant *p53* gene with a codon 72 polymorphism and a point mutation at codon 113 (T → G-phenylalanine to cysteine). The 5637 cell line contains a mutant *p53* gene with a codon 72 polymorphism and a point mutation at codon 280 (G → C-arginine to threonine), which has previously been reported as a mutational hot spot for bladder cancer. In the *p53* database, where over 10,000 *p53* mutations have been reported, codon 280 mutations make up 1.2% of all reported mutations but 5.1% of bladder cancer mutations [24, 25]. This mutation at codon 280 has been reported to be a dominant-negative mutation [28]. In the present study, to investigate the function of *p53* in the response of the five bladder cancer cell lines to ionizing radiation, we examined the expression levels of *p53* mRNA after ionizing radiation using real-time quantitative PCR assay.

Our data indicated that IR induced a significant increase in *p53* mRNA expression in KK47 and RT4 cells but not in T24, 5637, or UM-UC-3 cells and, consequently, induced a significant increase in the downstream *p21* and *Bax* gene effectors, which in turn caused cells to undergo G1 cell-cycle arrest and apoptosis. There are several reports suggesting that radiation-induced G1 cell-cycle arrest requires wild-type *p53* expression [29] and that G1 cell-cycle arrest may correlate with radiosensitivity [30, 31]. Pellegata et al. [32] found that when irradiated cells undergo wild-type *p53*-dependent G1 cell-cycle arrest, they do not subsequently arrest in G2, but that cells devoid of wild-type *p53* genes or containing mutations of the *p53* gene frequently show radiation-induced G2 cell-cycle arrest [11]. Consistent with these reports, KK47 and RT4 with wild-type *p53* gene in the present study showed radiation-induced G1 cell-cycle arrest with elevated expression of *p53* and *p21* genes, while the T24, 5637, and UM-UC-3 cells containing mutations of the *p53* gene showed radiation-induced G2 cell-cycle arrest.

The actual site of the mutation of the *p53* gene is important, since mutations in the DNA-binding domain have the greatest effect on function. Most *p53* mutations in cancers are located in this domain, and mutations here lead to the production of *p53* protein that fails to bind to DNA in the normal sequence-specific fashion. Different *p53* mutations induce different downstream effects; for example, certain mutants activate the *p21* gene but not *Bax*, and others affect the cell-cycle rather than apoptosis [33, 34]. In our experiment, neither *p21* nor *Bax* was activated in three *p53*-mutated cell lines after IR.

p53 is a very short-lived transcriptional activator that, when stabilized, can induce apoptosis [35], and that plays a key role in regulating apoptosis, especially in the setting of radiation exposure [36]. While transcription-independent mechanisms for *p53*-dependent apoptosis have been recognized, the apoptotic response of tumor cells to irradiation is thought to be closely related to the ability of *p53* to activate transcription [37]. The *Bax* gene has been shown to contain a *p53*-responsive element in its promoter [38]. This makes it an attractive candidate as a downstream effecter of *p53*-dependent apoptosis. Consistent with this possibility is the observation that some naturally occurring *p53* mutants that have selectively lost the ability to activate transcription of *Bax* also appear to have lost the ability to induce apoptosis in many cell types [25]. In the present study, TUNEL assay showed that IR-induced cell apoptosis with increased expression of *p53* and *Bax* genes only in KK47 and RT4 cells, while neither significant induction of cell apoptosis, nor elevation of *p53* or *Bax* gene expression were observed in T24, 5637, or UM-UC-3 cells after IR. Another study also reported the possible function of Bax in the signal transduction pathway to radiation-induced apoptosis in the human peripheral primitive neuroectodermal tumor cell line with wt-*p53* [39]. Furthermore, in a clinical study, Ito et al. have reported that Bax protein expression after chemoradiotherapy could be a significant prognostic marker for maxillary squamous cell carcinoma [40]. These data lend support to the increasing evidence that the expressions of *p53* and *Bax* may play a role in radiation-induced cell apoptosis and contribute to increased cell radiosensitivity.

Conclusion

We employed five different human bladder cancer cell lines, two expressing wt-*p53* and the other three containing mutations of the *p53* gene, to investigate the possible relationship between *p53* status and radiosensitivity in bladder cancer cells. Our observations indicated that the bladder cancer cell lines expressing wt-*p53* were more sensitive to IR than the three containing

mutations of the *p53* gene, possibly due to the *p53*-dependent radiation-induced cell apoptosis.

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