

Enhancement of the radiosensitivity of cervical cancer cells by overexpressing p73 α

Stephanie Si Liu,¹ Kelvin Yuen-Kwong Chan,^{1,2} Rebecca Ching-Yu Leung,¹ Helen Ka-Wai Law,³ Tsin-Wah Leung,¹ and Hextan Yuen-Sheung Ngan¹

Departments of ¹Obstetrics and Gynaecology, ²Pathology, and ³Paediatrics and Adolescent Medicine, Queen Mary Hospital, The University of Hong Kong, Hong Kong SAR, China

Abstract

Radiation therapy is the most effective therapy for cervical cancer in advanced stages. p53 plays a critical role in the cellular response to radiation-induced DNA damage. However, p53 function is often impaired in the presence of the oncoprotein E6 from human papillomavirus, which is often associated with the development of cervical cancer. p73, a p53 family member, is highly similar to p53, but is resistant to the degradation by human papillomavirus E6. In this study, we investigated the role of p73 α in relation to cellular radiosensitivity in the p53-impaired cervical cancer cells. Radiosensitivity and irradiation-induced apoptotic cell death were examined in the exogenous overexpressed p73 α - and p53-impaired cells. Our results showed that the endogenous p73 α expressed only in the radiosensitive cervical cancer C4-1 cells, but not in the radioresistant SiHa, Caski, and HeLa cells. Overexpression of exogenous p73 α by transfection in the radioresistant cells resulted in a significant increase of cellular sensitivity to radiation. Enhanced radiosensitivity in p73 α -transfected cells was attributed by increase of cellular apoptosis. Coactivation of p21 was also observed in the p73 α -transfected cells upon radiation treatment. In summary, our findings suggested that p73 α is an important determinant of cellular radiosensitivity in the p53-impaired cervical cancer cells. [Mol Cancer Ther 2006;5(5):1209–15]

Introduction

The p53 tumor suppressor protein is a key mediator of an ATM-dependent DNA damage response cascade following

cellular exposure to ionizing radiation. Abrogation of p53 function often leads to increase of radioresistance in many human tumors (1). Although p53 mutation in cervical cancers is rare with an overall incidence of 1% to 6% (2), the p53 function is impaired by human papillomavirus (HPV) oncoprotein in most of cervical cancers. HPV is found to integrate into the cellular DNA of the cervical epithelial cells, and nearly 90% of the cervical cancer cells are infected with HPV (3). The oncoprotein E6 of HPV forms complexes with p53 and promotes p53 degradation via the ubiquitin-dependent mechanism (4, 5). Although most of the cells lacking p53 function are less responsive to radiotherapy and/or chemotherapy, they are not completely resistant (6–8). There should be more than one alternative route leading to cancer cell apoptosis in response to DNA damages. Our previous study has shown an association between the p73 α expression and the cellular radiosensitivity in cervical cancers (9). The overexpression of p73 α was observed in radiosensitive cancers and had prognostic significance in predicting the outcome of the cervical cancer patients after radiotherapy. In addition, the epigenetic modification via promoter hypermethylation might be a critical alternative mechanism in inactivation of p73 expression in the cervical cancers.

p73 belongs to the p53 tumor suppressor family. Significant structural homology exists between p73 and p53. The full-length p73, mainly TAp73 α and TAp73 β , markedly mimics p53 function in experimental systems. It activates many p53 cellular target genes and is capable of inducing cell cycle arrest and apoptosis (10–12). Moreover, p73 can be induced in response to multiple DNA-damaging signals, such as γ -irradiation, or anticancer drugs like cisplatin, Taxol, and camptothecin (13–16). Several recent studies have revealed the importance of p73 gene in regulation of cellular sensitivity to chemotherapeutic drugs. Flores et al. (17) have shown that mouse embryo fibroblasts lacking p73, similar to those cells lacking p53, were more resistant to chemotherapy than their wild-type counterparts. Studies on human tumor cell lines have further confirmed that p73 is an important determinant of chemotherapeutic efficacy in human tumors. p73 can be induced by a wide variety of anticancer drugs, whereas blocking of p73 function led to chemoresistance of human tumor cells (15). Furthermore, p73 could functionally replace p53 in triggering apoptosis and cell cycle arrest or DNA repair effectors in Adriamycin-treated, p53-deficient breast cancer cells (18). Apparently, most of the studies were focused on p73 in relation to chemosensitivity, yet very little work has been published documenting the role of p73 in the radiosensitivity of human cancers. In the present study, we used an *in vitro* cell model to investigate the role of p73 in regulating the cellular sensitivity to radiation treatment in cervical cancer.

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Requests for reprints: Hextan Yuen-Sheung Ngan, Department of Obstetrics and Gynaecology, Queen Mary Hospital, 6th Floor, Professorial Block, Pokfulam Road, Hong Kong SAR, China. Phone: 852-28554684; Fax: 852-28550947. E-mail: hysngan@hkucc.hku.hk

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Materials and Methods

Cell Culture and Radiation Treatment

The human cervical cancer cell lines C4-1 (HPV 18⁺), HeLa (HPV 18⁺), SiHa (HPV 16⁺), and Caski (HPV 16⁺) were grown in 10% fetal bovine serum and 1% penicillin and streptomycin-supplemented MEM (Life Technologies, Inc., Gaithersburg, MD).

The cells were detached from culture flasks and washed with PBS. They were then suspended in culture medium and irradiated at single doses of 2 and 6 Gy, respectively. Irradiation of cells was done using a gamma irradiator MDS Gammacell 3000 Elan (Elitemodel D, Nordion International, Inc., Ottawa, Canada).

p73 α Expression Construct and Cell Transfection

The wild-type p73 α transcript was amplified from the HT29 cell line. Primers were designed to amplify the complete coding region of the p73 α forward: 5'-AAGA-TGGCCAGTCCACCGCC-3' and reverse 5'-GTGGAT-CTCGGCTCCGTA-3' using the Expand High Fidelity Plus PCR System (Roche Applied Science, Indianapolis, IN) according to the instructions of the manufacturer. The amplified fragment was cloned into pcDNA3.1 expression vector (Invitrogen). The pcDNA3.1/p73 α clone was subsequently confirmed by direct sequencing.

Culture cells were seeded 1 day before the transfection. Transfection was carried out using FuGene 6 transfection reagent (Roche Applied Science) according to the recommendation of the manufacturer. The cells were transfected with pcDNA3.1/p73 α or with the vector pcDNA3.1 alone as mock transfection. After 24 to 48 hours of incubation, they were harvested and then subjected to irradiation.

Quantitative p73 α mRNA Expression Analysis

Total RNA was isolated from the cell lines using TRIzol reagent (Life Technologies) and reverse transcribed to cDNA using Superscript III Reverse Transcriptase (Life Technologies). Real-time quantitative PCR was done to quantify the expression level of p73 α in the synthesized cDNA using the p73 α sequence-specific primers and TaqMan probe: (forward) 5'-GGCTGCGACGGCTGCAGAGC-3' (reverse), 5'-TCAGCAGATTGAACTGGGCCATGACAGAT-3', and (probe) 5'-FAM-CGTTCCGCCACCACCT-CAT-TAMRA-3'. An endogenous control, TBP, was used to normalize the expression of p73 α . The standard curve was set up using three serial 10-fold diluted linearized plasmids containing the p73 α or TBP cDNA insert. All measurements were conducted in triplicates.

Immunoblotting Analysis

Proteins were extracted from the cells using the conventional method. The immunoblotting was done using antibodies of mouse anti-p73 (specific for p73 α , Zymed, San Francisco, CA), anti-p21^{WAF1} (Calbiochem, Boston, MA), and anti-Bax (Neomarkers, Lab Vision Corporation, Fremont, CA) in a dilution of 1:500. The sheep anti-mouse secondary antibody labeled with horseradish peroxidase (Amersham, Arlington Heights, IL) was subsequently applied. The signals were visualized with the ECL chemiluminescent detection kit (Amersham) and autoradi-

ography. The membranes were also reprobbed with β -actin (Sigma Co., St. Louis, MO) after stripping.

Clonogenic Survival Assay

The clonogenic assay was done on single cell suspension. Following irradiation, cells were plated as 1,000 per well in a six-well plate and incubated for 10 to 14 days. Colonies that consisted of >50 cells were scored after fixed with 70% ethanol and stained with 1% Giemsa solution. At least three independent experiments were done for each cell line. The intrinsic radiosensitivity of cells was determined by the survival fraction at 2 Gy (SF₂), as cell survival following a single *in vitro* dose (2 Gy) of irradiation (19). Cells with SF₂ < 0.4 were considered to be radiosensitive, but those with SF₂ > 0.4 were resistant. The ID₅₀, a dose that inhibited 50% of the colony-forming ability compared with the untreated control, was also calculated to compare the radiosensitivity between the cell lines.

Apoptosis Assay

The cell apoptosis was assessed by the detection of caspase-3 activity using the ApoAlert Caspase-3 Colorimetric Assay kit (BD Biosciences Clontech, Palo Alto, CA) according to the instructions of the manufacturer. The assay was done on both irradiated and nonirradiated cells (5 × 10⁶), which were cultured for 72 hours after irradiation. Colorimetric intensity was measured using Dynatech MR 5000 plate reader (Dynatech Laboratory Ltd., Billingham, United Kingdom) at a wavelength of 405 nm. The caspase-3 activity in cells was quantified by a standard curve established with free chromophore *p*-nitroaniline. HL-60 cells treated with camptothecin were used as positive controls.

A flow cytometry-based assay using the Vybrant Apoptosis Assay kit #4 (Molecular Probes, Leiden, the Netherlands) was also used to quantitatively examine the apoptotic cell death according to the specifications of the manufacturer. This assay was based on the differential permeability of the apoptotic and dead cells to the green fluorescent dye (YO-PRO-1) and the red fluorescent dye [propidium iodide (PI)], respectively. Cells were harvested 72 hours after irradiation and suspended at 1 × 10⁶/mL in PBS. After staining with YO-PRO-1 and PI for 30 minutes, cells were analyzed by flow cytometry (COULTER EPICS ELITE, Beckman Coulter Corporation, Miami, FL). Ten thousand events per sample were collected into listmode files and the percentages of intact cells in apoptosis (YO-PRO-1⁺/PI⁻) and necrosis (YO-PRO-1⁻/PI⁺) were determined.

Statistical Analysis

The experimental results were expressed as means ± SE. Analysis was done using one-way ANOVA and unpaired Students' *t* test wherever appropriate. *P* < 0.05 was considered statistically significant.

Results

p73 α Expression Was Associated with the Radiosensitivity of Cervical Cancer Cells

We studied whether p73 α expression was correlated with the *in vitro* radiosensitivity of cervical cancer cells. Both mRNA and protein expressions of p73 α were assessed in

four cervical cancer cell lines. The p73 α expression was found in C4-1 cells only, but not in HeLa, Caski, and SiHa cells (Fig. 1A and B).

The *in vitro* radiosensitivity of the four cell lines was determined by clonogenic survival assay, and their radiation survival patterns were shown in Fig. 2. Among the four cell lines studied, C4-1 was the most sensitive to irradiation. The survival of C4-1 cells began drastically decreasing from 2 Gy irradiation. When ID₅₀ was calculated and compared among the four cell lines, C4-1 cells had the lowest value (1.51 ± 0.05), whereas HeLa had the highest value (2.13 ± 0.07 ; Table 1). A significant difference was observed between C4-1 and any of the other three cell lines ($P < 0.001$). The intrinsic radiosensitivity of these cell lines, determined by the survival fraction at 2 Gy (SF₂), ranged from 0.25 (C4-1) to 0.67 (HeLa; Table 1). C4-1 cells were also highly sensitive to irradiation as they were found to have significant lower SF₂ value (0.25 ± 0.03) when compared with the SF₂ values of the other three cell lines (all above 0.6, $P < 0.001$). Both ID₅₀ and SF₂ analyses yielded consistent findings and thus indicated that C4-1 seemed to be radiosensitive, whereas HeLa, Caski, and SiHa were radioresistant.

Overexpression of Exogenous p73 α Increased the Radiosensitivity of Cervical Cancer Cells

pcDNA3.1/p73 α was transfected into HeLa, Caski, and SiHa cell lines, which lacked endogenous p73 expression. After 24 to 48 hours of incubation, the exogenous p73 α was constantly expressed in these three cell lines with and without irradiation (2 Gy; Fig. 1C and D). The endogenous p73 expression was absent in the mock and nontransfected cells whether or not they received radiation (2 Gy; Fig. 1C and D).

The clonogenic assay was done to assess the cell survival after the transfection in combination with radiation treatment. Upon the radiation treatment, marked reduction of cell survival was observed in the p73 α -transfected HeLa, Caski, and SiHa cells (Fig. 3A–C). ID₅₀ of the p73 α -transfected HeLa and Caski cells significantly

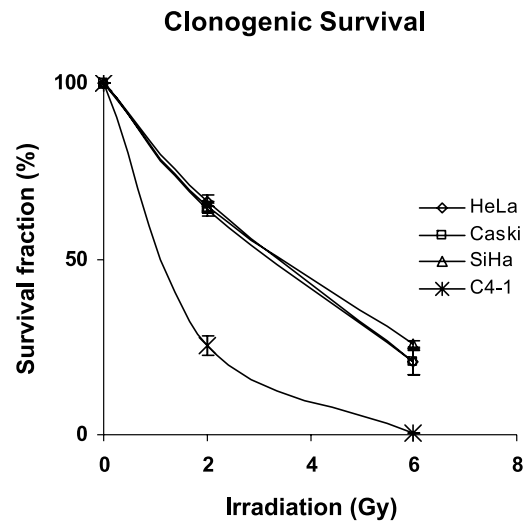


Figure 2. The clonogenic survival of four cell lines following irradiation. The colony-forming ability was calculated by comparing the numbers of colonies between the irradiated and nonirradiated controls. Points, means of three independent experiments; bars, SE. Gy, irradiation dose.

decreased from 2.13 to 1.80 ($P < 0.01$) and 2.07 to 1.77 ($P < 0.05$), respectively, and a trend of reduction from 2.04 to 1.89 ($P > 0.05$) was observed in p73 α -transfected SiHa cells (Table 2). Survival fraction at 2 Gy (SF₂) was measured and the radiosensitivity of cells was then determined (Table 2). SF₂ of the p73 α -transfected HeLa, Caski, and SiHa cell lines significantly decreased from 0.67 to 0.40 ($P < 0.001$), 0.65 to 0.38 ($P < 0.001$), and 0.65 to 0.50 ($P < 0.05$), respectively. In addition, our results showed that transfection alone would only slightly affect cell survival (data not shown).

p73 α Overexpression Enhanced Cancer Cell Apoptosis following Irradiation

The contribution of overexpressing p73 α toward radiation-induced apoptosis was further investigated. Caspase-3 activity results showed that radiation-induced apoptosis was significantly higher in the p73 α -transfected cells than that in the control cells (nontransfected cells or mock transfection control, $P < 0.05$; Fig. 4A). In concordance with the caspase-3 result, the percentage of the YO-PRO-1-positive apoptotic cells was significantly

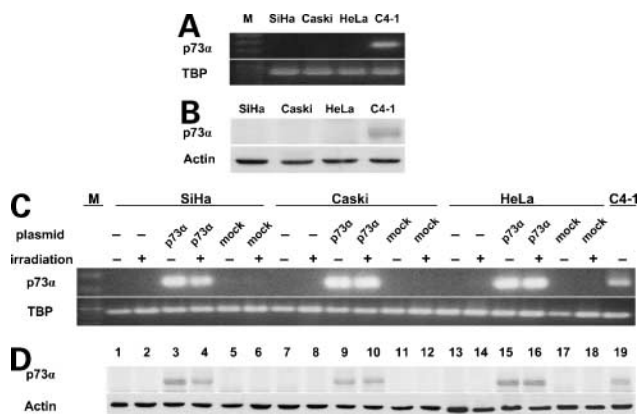


Figure 1. Detection of the p73 α expression in four cervical cancer cell lines by real-time quantitative reverse transcription-PCR (A) and immunoblotting (B). Induction of the p73 α mRNA (C) and protein expressions (D) in HeLa, Caski, and SiHa cells after gene transfection (one representative quantitative reverse transcription-PCR results).

Table 1. Radiosensitivities of four cervical cancer cell lines analyzed by ID₅₀ and SF₂

| Cell line | ID ₅₀ | SF ₂ |
|-----------|-------------------|-------------------|
| C4-1 | $1.51 \pm 0.05^*$ | $0.25 \pm 0.03^*$ |
| SiHa | 2.04 ± 0.03 | 0.65 ± 0.02 |
| Caski | 2.07 ± 0.06 | 0.64 ± 0.02 |
| HeLa | 2.13 ± 0.07 | 0.67 ± 0.02 |

NOTE: Average measured values with SE were calculated from three independent experiments.

* $P < 0.001$, one-way ANOVAs between C4-1 and any other cell lines.

Table 2. Decrease of cell survival (ID₅₀ and SF₂) in the p73 α -transfected cervical cancer cell lines

| Cell line | ID ₅₀ | ANOVA* | SF ₂ | ANOVA* |
|----------------------|------------------|-----------------|-----------------|------------------|
| HeLa | 2.13 \pm 0.07 | | 0.67 \pm 0.02 | |
| HeLa mock | 2.10 \pm 0.07 | | 0.63 \pm 0.01 | |
| HeLa + p73 α | 1.80 \pm 0.09 | <i>P</i> < 0.01 | 0.40 \pm 0.07 | <i>P</i> < 0.001 |
| Caski | 2.07 \pm 0.06 | | 0.64 \pm 0.02 | |
| Caski mock | 2.07 \pm 0.04 | | 0.65 \pm 0.02 | |
| Caski + p73 α | 1.77 \pm 0.04 | <i>P</i> < 0.05 | 0.38 \pm 0.03 | <i>P</i> < 0.001 |
| SiHa | 2.04 \pm 0.03 | | 0.65 \pm 0.02 | |
| SiHa mock | 1.92 \pm 0.05 | | 0.61 \pm 0.03 | |
| SiHa + p73 α | 1.89 \pm 0.02 | <i>P</i> > 0.05 | 0.50 \pm 0.02 | <i>P</i> < 0.05 |

*One-way ANOVAs between the p73 α -transfected (+p73 α) and the mock-transfected or nontransfected control cells.

higher in the irradiated p73 α -transfected cells (*P* < 0.05) when compared with the irradiated mock-transfection controls (Fig. 4B and C). On the other hand, the number of the necrotic cells was only slightly increased upon irradiation in both p73 α -transfected and mock-transfection control cells (data not shown).

Transactivation of p21 in the p73 α -Transfected Cells

The protein expressions of p21 and Bax were assessed and shown in Fig. 5. The expression of p21 was found in Caski cells, but not in SiHa and HeLa cells. In response to irradiation, the expression of p21 was up-regulated in all p73 α -transfected cells, but not in the mock and the nontransfected cells. On the other hand, the expression of Bax was found constantly expressed throughout, irrespective of irradiation (Fig. 5).

Discussion

Over the past several years, many studies showed that p73 could be activated in response to a subset of DNA-damaging agents. The activation pathway of p73 in response to genotoxic stress was distinct from that of p53 and was linked to the c-abl tyrosine kinase, which phosphorylated and activated p73 (14, 20). Accumulated evidences suggested that p73 was essential for apoptosis induced by irradiation and many chemotherapeutic agents (15, 17). The induction of apoptosis by p53 required the presence of p73, whereas p73 was able to induce apoptosis in the absence of p53. A recent study has linked the chemosensitivity of cancer cells to the function of p73 irrespective of p53 status (15). In the present study, p73 α expression was detected only in radiosensitive C4-1 cell line, but not in radioresistant SiHa, Caski, and HeLa cell lines. With overexpressing exogenous p73 α , the latter three cell lines had significantly increased of sensitivity to radiation. These results were in concordance with our previous study done in clinical cervical cancer whose clinical outcome to radiotherapy was associated with the p73 α expression (9). It thus suggested that p73 α might play an important role in the regulation of cellular response to radiation in cervical cancers.

Enhanced radiosensitivity in the p73 α -transfected cells was attributed by the increase of cell apoptosis. Apoptosis seemed to be the dominant mode of cell death in response to radiation in the studied cell lines. Only mild increased number of necrotic cells was observed in both p73 α -transfected and nontransfected control cells after irradiation. This radiation-induced and p73-dependent apoptotic pathway was likely to be p53-independent because the p73 α -transfected cell lines contained HPV high-risk types of 16 (SiHa and Caski) and 18 (HeLa). Unlike p53, p73

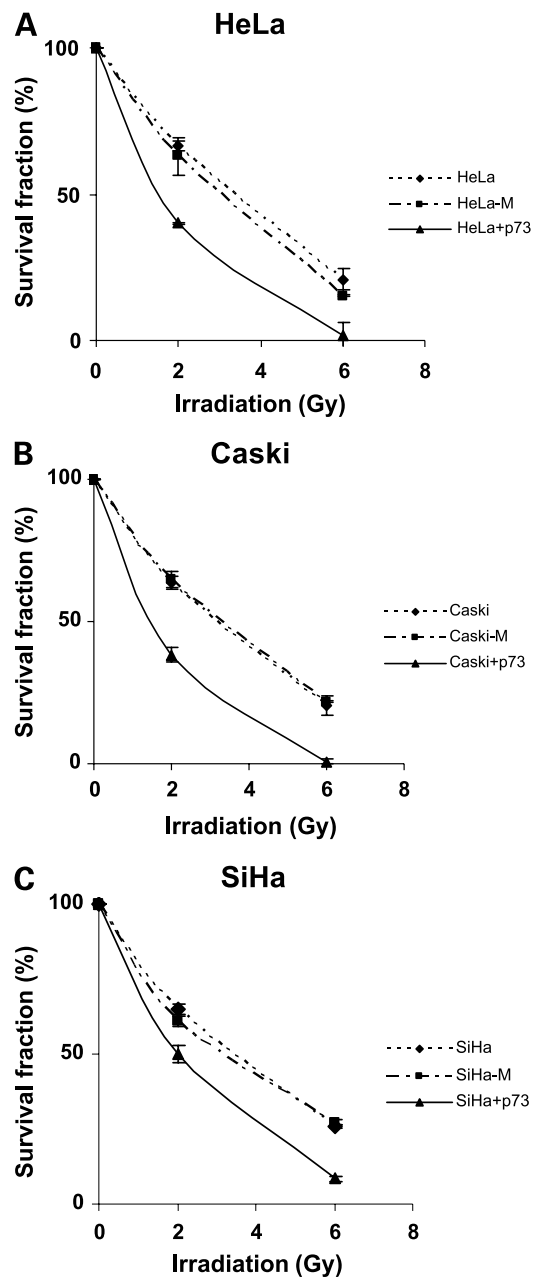


Figure 3. Overexpression of exogenous p73 α in HeLa (A), Caski (B), and SiHa (C) cells reduced the cell clonogenic survival following irradiation.

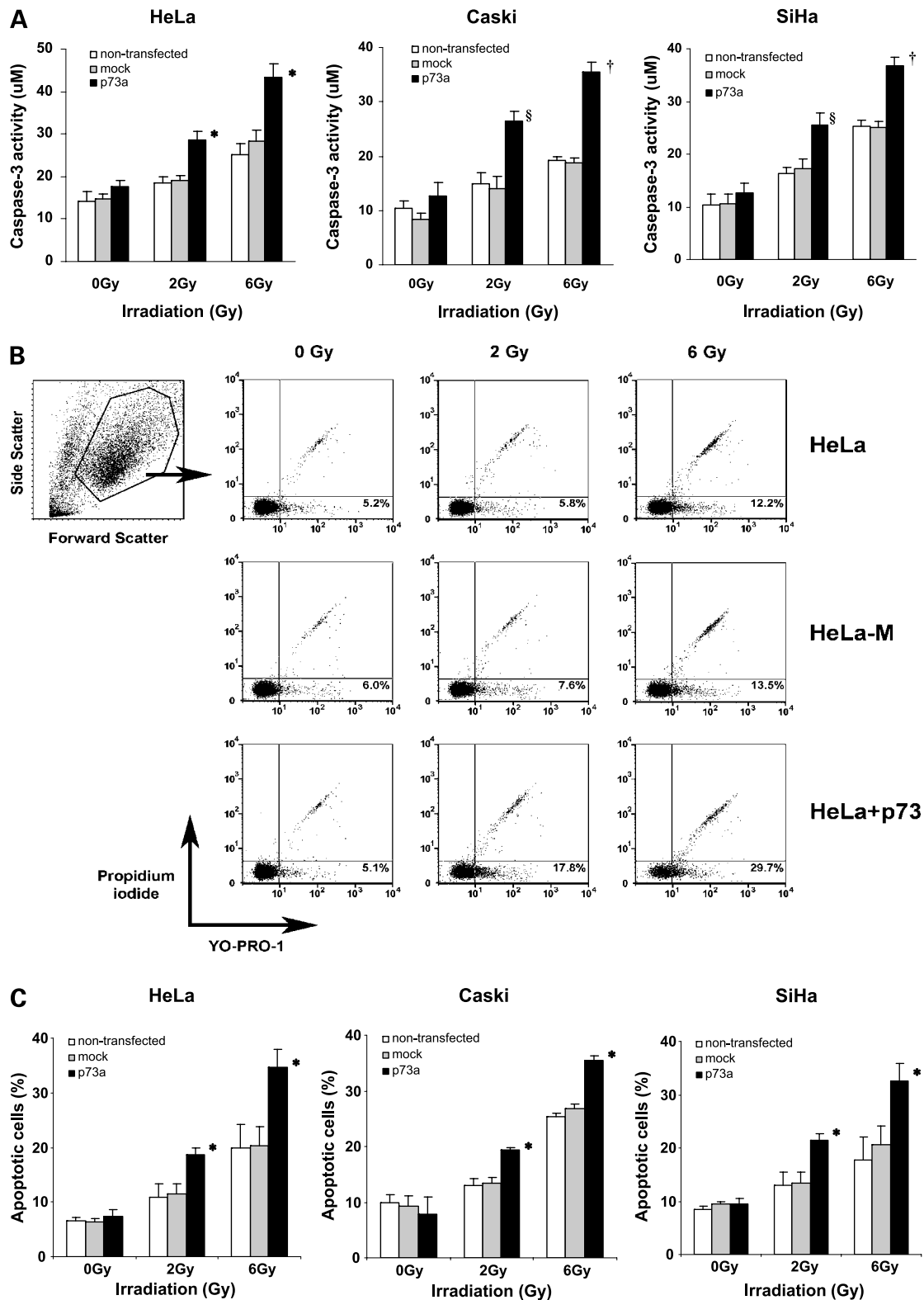


Figure 4. Overexpression of exogenous p73 α increased radiation-induced apoptosis. **A**, caspase-3 activity was significantly elevated in the p73 α -transfected cell lines. *, $P < 0.05$; §, $P < 0.01$; †, $P < 0.001$ versus the mock-transfected and the nontransfected control cells. **B**, the percentage of cell death via apoptosis (YO-PRO-1⁺/PI⁻) and necrosis (YO-PRO-1⁻/PI⁺) were analyzed by flow cytometry. Representative results of HeLa cells from three independent experiments. **C**, similar pattern of increased radiation-induced apoptosis was detected in all three cell lines. Columns, mean; bars, SE ($n = 2/3$). *, $P < 0.05$.

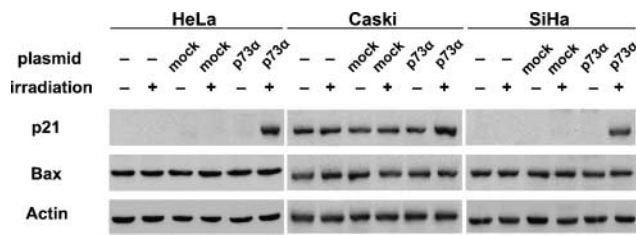


Figure 5. Differential expressions of p21 and Bax in the p73 α -transfected and the nontransfected cell lines following radiation treatment.

could not be targeted and inactivated by the HPV oncoprotein E6 physically or functionally (21). It had been shown that p73 would up-regulate the p21 expression in cells that underwent apoptosis after treatment with DNA-damaging agents (11). Increase of p21 expression was also observed in our p73 α -transfected cervical cancer cells of which they were found highly sensitive to radiation when compared with the mock-transfected and nontransfected control cells. This result suggested that the increase of p21 expression in the p73 α -transfected cells was p53 independent because the absent or unchanged p21 expression was found in the mock-transfected and nontransfected cells following irradiation. The Bax expression, however, expressed constantly irrespective of the radiation treatment. The current findings of the differential expressions of p21 and Bax were consistent with our previous observations from clinical cervical cancer samples in which the p21 expression, but not the Bax expression, was positively correlated with the p73 α expression (9). Although the transactivation of Bax via p73 was still controversial in other studies (11, 22, 23), our results, coupled with the expression patterns of p21 and Bax, suggested that p73 would induce apoptosis through other mechanisms, which might be different from its homologue p53. Further studies on p73 downstream genes may shed light on the p73 function in regulating the cellular response to radiation.

A p73 α knockdown assay using p73 α -specific small interfering RNA was also done on the radiosensitive C4-1 cells; however, the transfection efficiency was very low. As much as 10% to 20% of transfection efficiency could only be achieved using various brands of commercially available transfection reagents. Nevertheless, there was a trend of decreased p73 α expression in quantitative analysis after small interfering RNA transfection. No obvious change was observed in the subsequent clonogenic survival assay and apoptosis assays (data not shown). C4-1 was the only cervical cancer cell line found to be sensitive to radiation treatment in our laboratory. Establishing a stable p73 α -specific small interfering RNA-transfected C4-1 cell line model would be an alternative method to efficiently knockdown the endogenous p73 α expression for future study.

In this study, no induction of the endogenous p73 α expression was found in the irradiated cervical cancer cells. A previous study showed that radiotherapeutic treatments

had no effect on altering the endogenous p73 protein levels in cancer cells (24). It has been suggested that the expressions of p73 and p53 were induced by different signals and play fundamentally different roles with respect to the maintenance of cell homeostasis (10). Unlike p53, radiation activated p73 through the tyrosine kinase c-abl-mediated phosphorylation and not by stabilization of the p73 protein (13, 20). Moreover, the response of p73 to DNA damage was highly dependent on the cellular context. Some other factors, such as the expression status of the endogenous antiapoptotic or proapoptotic genes and the epigenetic mechanisms, might also influence the activation and the ability of p73 α to induce apoptotic cell death (25).

Down-regulation of p73 expression was found to be significantly correlated with epigenetic promoter methylation or allelic loss (12). The methylation-dependent silencing of p73 α in clinical cervical cancer samples was previously shown to have significant association with the adverse outcome of the patients following radiotherapy (9). The three radioresistant cell lines included in this study were also found to have p73 α promoter hypermethylation, but such hypermethylation pattern was not observed in the radiosensitive C4-1 cells (9). The demethylation agent, 5-aza-2'-deoxycytidine, was able to restore p73 α expression in the three radioresistant cell lines. However, the drug treatment was a nonspecific process that could globally reactivate other genes suppressed by promoter methylation; hence, the demethylation drug-treated cell line model might not account for the effect of p73 α in response to radiation.

In summary, we were able to show that increase of p73 α expression would result in an increase of irradiation-induced apoptotic cell death in our *in vitro* cell model. This strongly suggested that p73 α played a very important role in regulating the cellular response to radiation, and it provided an alternative radiation-induced apoptosis pathway, especially in cervical cancers where most of which were lacking of functional p53. Therefore, novel treatment strategy to increase the cancer cell sensitivity to radiotherapy by modulation of the p73 α expression could be developed for cervical cancers.

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