

## Phosphorylation of p53 Modifies Sensitivity to Ionizing Radiation

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**Abstract.** *Phosphorylation is an important modification involved in the control of p53 activity. We examined the relationship between p53 phosphorylation and cell radiosensitivity. We prepared H1299 cells (p53-null) with various mutations of p53 at three sites (serine 15, 20 and 46) and examined the radiosensitivity of the cells. In three mutant forms of p53 – S15A, S20A and S46A – serine was converted to alanine at these sites to prevent phosphorylation, and in two other mutant forms, S15D and S20D, serine was converted to aspartic acid to mimic phosphorylation. H1299 cells were more radioresistant than cells with wild-type p53. Cells with the S15A and S46A mutant forms of p53 were radiosensitive, whereas those with the S15D, S20A and S20D forms showed medium radiosensitivity. Thus the sensitivity of cells to ionizing radiation varies according to the site of phosphorylation of p53.*

It has been well established that p53 plays multiple tumor-suppressive roles in cells upon introduction of stress. The p53 protein accumulates after exposure to ionizing radiation and triggers arrest of the cell cycle or promotion of apoptosis (1-3). In irradiated cells, the p53 protein can enhance cell survival through DNA repair during growth arrest or decrease it by induction of apoptosis. Structural and functional analyses of p53 have shown that it is a transcription factor with a sequence-specific DNA-binding domain in the central region and a transcriptional activation domain at the N-terminal. The p53 protein is phosphorylated at multiple sites at the N- and C-terminals by a number of

kinases. Ataxia telangiectasia mutated (ATM) family kinases are required for the rapid phosphorylation of human p53 at serine 15 or other N-terminal serine sites following damage to DNA by ionizing radiation (4-6). A number of p53 phosphorylation events after DNA damage have been identified, and the potential roles of these events in regulating the stability or activity of p53 have been examined (7, 8). We hypothesized that phosphorylation of p53 may affect the radiosensitivity of cells. To examine the effect of phosphorylation on radiosensitivity, we prepared several mutant forms of p53 at the N-terminal serine residues 15, 20 and 46, and transferred these mutant p53 forms to the human non-small cell lung carcinoma cell line, H1299, which is null for p53.

### Materials and Methods

**Cell culture.** The H1299 cell line (human non-small cell lung carcinoma, p53-null) was cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). The transformants were selected in medium containing 100 µg/ml hygromycin or 400 µg/ml G418.

**Plasmids.** The LacSwitch inducible mammalian expression system (Stratagene, La Jolla, CA, USA) was used. The expression plasmid was pOPI3CAT, and the CAT gene was replaced by a mutant or wild-type p53 gene at the *NotI* site. H1299 cells were transfected with 2 µg of expression plasmid using the calcium phosphate precipitation method (9). A plasmid p3'SS, expressing the *lac* gene encoding the lac repressor protein, was used as a regulation plasmid. The expression plasmid and regulation plasmid were then co-transfected into the cells. The expression of each mutant p53 gene was suppressed by the lac repressor in this LacSwitch system. IPTG at 5 mmol in the medium induced the expression of each mutant p53 in the cells.

**In vitro mutagenesis.** Mutagenesis was performed with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) in accordance to the manufacturer's protocol. Wild-type p53 cDNA (pPro Sp53: CD 104; Japanese Tumor Research Resources Bank) was introduced into the M13 vector. Oligonucleotides for the mutations were ordered from Hokkaido System Science, Japan. The mutant p53 DNA sequences were checked with a DNA sequencing

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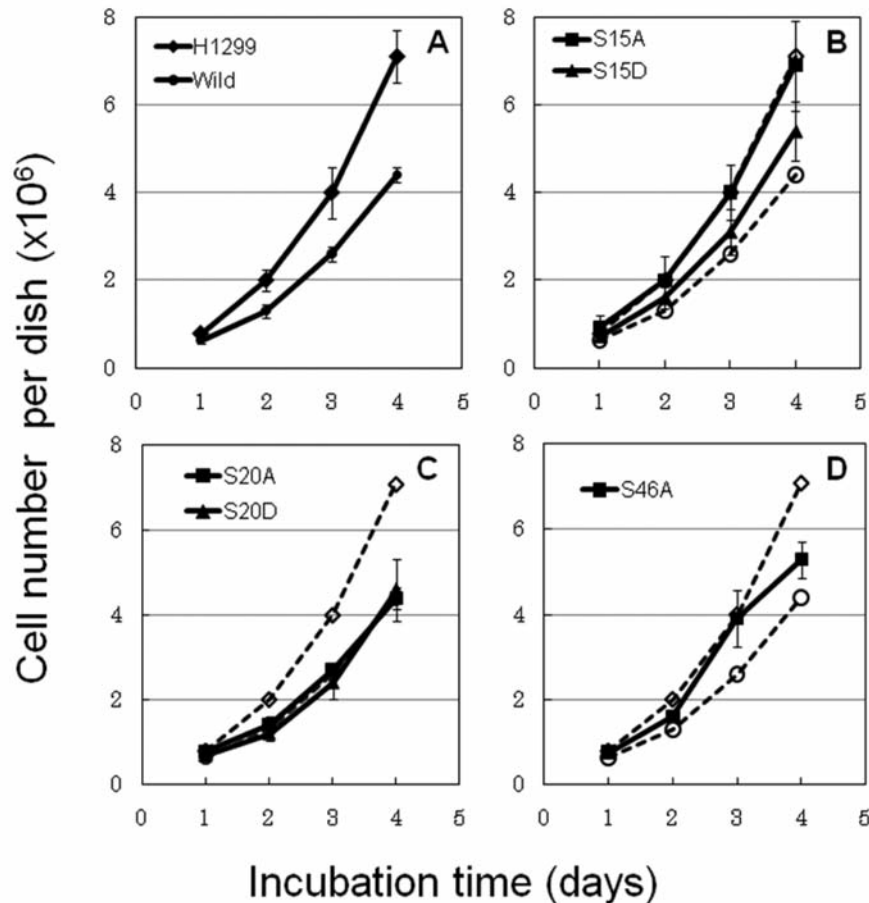


Figure 1. Inhibition of growth in mutant *p53* cells. H1299, the wild-type *p53* transformant and 5 mutant *p53* transformants were incubated for 5 days with 5 mmol IPTG. In B, C and D, the growth curves of the wild-type *p53* transformant (circle) and H1299 (rhombus) are indicated by dashed lines. Error bars represent standard error.

kit and a model 373A DNA sequencer (Applied Biosystems, Van Allen Way, CA, USA). We produced two types of *p53* mutation. In one type, serine was replaced by alanine at positions 15, 20 and 46 to create S15A, S20A and S46A. In these mutant forms of *p53*, phosphorylation does not occur at these positions. In the other type, serine was replaced by aspartic acid at positions 15 and 20 to create the S15D and S20D mutants, in which phosphorylation is mimicked at these positions.

**Western blotting of *p53* protein.** Total protein was extracted as described previously (10), and 30  $\mu$ g of protein was subjected to Western blot analysis. Anti-*p53* monoclonal antibody (Pab 1801, Santa Cruz Biotechnology, CA, USA) was used. Protein levels were analyzed using enhanced chemiluminescence (Amersham, Piscataway, NJ, USA).

**Radiation treatment and survival studies.** After addition of 5 mmol IPTG and culture in DMEM supplemented with 10% FCS for one day, cells were irradiated with a PS-3100S Cs-137  $\gamma$ -ray machine (Pony Industry Co. Ltd., Osaka, Japan) at a dose of 0.993 Gy/min at room temperature. The cells were trypsinized before irradiation. After irradiation, the cells were plated onto 10-cm dishes at

densities expected to yield about 50 clonogenic cells. Colonies were examined after about two weeks to calculate the surviving fraction. Three replicate plates per experiment were used for each survival point, and the experiments were repeated at least three times. The survival results were calculated as mean  $\pm$  SE, and comparisons between values were analyzed statistically by Student's *t*-test.

## Results and Discussion

We cloned H1299 cells harboring mutant *p53* (S15A, S15D, S20A, S20D, S46A) and wild-type *p53*. Western blotting was performed to determine the expression of *p53* protein in the cloned cells. Phosphorylation of *p53* modifies the transcriptional activity of various genes (7). First, we examined the cell growth inhibition of each mutant resulting from phosphorylation of *p53*. The cells at  $5 \times 10^5$  per dish (100 $\times$ 20 mm style Falcon tissue culture dish) were incubated with 5 mmol IPTG for induction of *p53* and cell numbers were checked each day for 5 days.

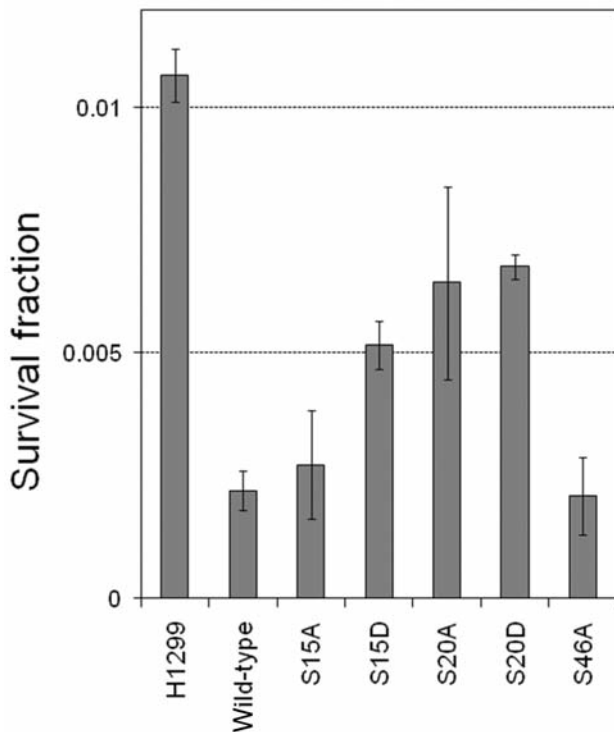


Figure 2. Radiosensitivity of mutant *p53* cells. H1299, the wild-type *p53* transformant, and 5 mutant *p53* transformants were exposed to 10 Gy of radiation. Error bars represent standard error.

H1299 cells grew to  $7.1 \times 10^6$  cells/dish, whereas the cells with wild-type *p53* grew to only  $4.4 \times 10^6$  cells/dish at 5 days (Figure 1A). This indicates that wild-type *p53* suppressed cell growth in this system. The cells harboring the S20A and S20D *p53* mutants also showed cell growth suppression, similar to the cells harboring wild-type *p53* (Figure 1C). On the other hand, the cells harboring S15A *p53* grew as well as H1299 cells (Figure 1B). These results indicated that phosphorylation of *p53* at serine 15, but not at serine 20, must be important for suppression of cell growth. The cells harboring the S15D *p53* mutant showed partial growth suppression (Figure 1B). Replacement of serine by aspartic acid at position 15 appears to mimic phosphorylation of serine. Duman and Meek (11) reported that substitution of alanine at position 15 resulted in a reduction in the capacity of *p53* to transactivate the *WAF1* promoter, while *p53* encoding aspartic acid at this position retained its transactivation function. Our data corresponds well with their report (Figure 1B).

Cells harboring the S45A mutant *p53* showed moderate growth suppression (Figure 1D). Therefore we considered that phosphorylation of *p53* at serine 45 may affect cell growth to some degree.

We then examined the radiosensitivity of the various transformants. Figure 2 shows the survival fractions of each of the mutant cell types after exposure to 10 Gy of radiation. The parental H1299 cells were radioresistant, but the wild-type *p53* transformant was radiosensitive ( $p < 0.005$ ). The S15A and S46A transformants were as radiosensitive as the wild-type *p53* cells ( $p > 0.05$ ). On the other hand, the S15D, S20D ( $p < 0.005$ ) and S20A ( $p < 0.01$ ) transformants exhibited more radioresistance than wild-type *p53* cells. As they were significantly more radiosensitive than H1299 cells ( $p < 0.005$ ), the S15D, S20A and S20D transformants had intermediate radiosensitivity. The data for S20A and S20D indicate that phosphorylation of *p53* at Ser20 must be involved in cell radiosensitivity. We considered that phosphorylation of Ser 15 is important for cell growth, but not for radiosensitivity, because the S15A transformant was as radiosensitive as cells with wild-type *p53*. However, the intermediate radiosensitivity of the S15D transformant is difficult to explain. Gorspe *et al.* (12) have reported that *WAF1* expression protects cells against *p53*-mediated apoptosis. Therefore we speculated that the S15D transformant induced *WAF1*, resulting in suppression of cell growth, and that this *WAF1* induction might also suppress apoptosis to some degree. The sensitivity of cells to ionizing radiation varied with the phosphorylation site at which *p53* was mutated. Few reports have described the radiation sensitivity of cells with mutation at phosphorylation sites of *p53*. MacPherson *et al.* (13) reported that B-cell lymphomas in mice with a *p53* point mutation at Ser 23 (corresponding to Ser 20 of human *p53*) showed radiosensitivity that was intermediate between wild-type and double-knockout *p53* cells. Their data corresponded well with our finding that S20A transformant cells had medium radiosensitivity. Oda *et al.* (14) reported that phosphorylation of Ser 46 was important for induction of apoptosis upon exposure to high-dose  $\gamma$ -ray irradiation (30 Gy). In the present study, the S46A transformant was radiosensitive to 10 Gy, suggesting that phosphorylation at Ser 46 is not important for induction of apoptosis, except upon exposure to a high radiation dose.

The tumor suppressor gene *p53* plays an important role in radiosensitivity. We have already reported that the sensitivity of cells to ionizing radiation varies with the mutation point within the DNA-binding region of *p53* (15). The *p53* gene is the most commonly mutated tumor suppressor gene in human tumors, being mutated in over half of all human tumors and the mutation points being widespread throughout the gene (16). However, reports describing mutation at phosphorylation sites in tumor cells have been scarce. In the present study, we showed that mutation at phosphorylation sites in the N-terminal region of *p53* led to variation in cell radiosensitivity. Currently it is unclear why mutation at phosphorylation sites is rare in tumor cells, and the mechanism of radiosensitivity may not be directly dependent on *p53*-mediated tumor suppression.

Recently it has been reported that many kinds of microRNAs related to tumorigenesis are regulated by p53 (17, 18). Thus p53 regulates not only mRNA but also microRNA, and these forms of regulation must be affected by the phosphorylation of p53. The available data suggest the importance of determining the phosphorylation of p53 in individual tumor cells in the context of radiotherapy.

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