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# Time-course analysis of DNA damage response-related genes after *in vitro* radiation in H460 and H1229 lung cancer cell lines

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Abbreviations: DDR, DNA-damage response; NSCLC, non small cell lung cancer

## Abstract

**Radiation is the most useful treatment modality for cancer patients. It initiates a series of signal cascades such as DNA damage response (DDR) signaling for repairing damaged DNA, arresting the cell cycle, and inducing cell death. Until now, few genes have been found to be regulated by radiation, which explains the molecular mechanisms of cellular responses to radiation. Although the transcriptional changes caused by radiation have been widely investigated, little is known about the direct evidence for the transcriptional control of DDR-related genes. Here, we examined the radiosensitivity of two non-small cell lung cancer cell lines (H460 and H1299), which have different p53 status. We monitored the time-dependent changes of 24 DDR-related gene expressions via microarray analysis. Based on the basal expression levels and temporal patterns, we further classified 24 DDR-related genes into four subgroups. Then, we also addressed the protein levels of several DDR-related genes such as TopBP1, Chk1 and Chk2, confirming the results of microarray analysis. Together, these results indicate that the expression patterns of DDR-related genes are associated with radiosensitivity and with the p53 statuses of H460 and H1299, which adds to the understanding of the complex biological responses to radiation.**

**Keywords:** carcinoma, non-small-cell lung; DNA damage; gene expression profiling; microarray analysis; radiation; tumor suppressor protein p53

## Introduction

Radiation therapy has been regarded as an effective treatment for various cancers. It causes DNA double strand breaks, base damage and DNA-protein crosslink to increase genomic instability. Subsequently, it leads to cell cycle arrest and cell death. To compensate for these cytotoxic events, cells recognize DNA damage and activate DNA repair system, in which DNA damage response (DDR) signaling plays a pivotal role. Upon DNA damage by ionizing radiation, DDR signaling promptly activates sensor kinases, ATM and ATR. The kinases firstly phosphorylate downstream kinases, Chk1 and Chk2 (Falck *et al.*, 2005). Then, these ultimately activate the numerous cellular responses involved in cell cycle arrest and DNA repair (Durocher and Jackson, 2001; Bartek and Lukas, 2003; Shiloh, 2003; Stracker *et al.*, 2009).

Aberrantly and constitutively activated DDR signaling is often observed in cancers (Bartkova *et al.*, 2006; Bartkova *et al.*, 2007). For example, γH2AX and the activation of ATM-Chk2-p53 pathway revealed that DDR machinery is constitutively activated in gliomas (Bartkova *et al.*, 2010). Possibly, aberrantly activated DDR signaling could be associated with radioresistance since the activation of DDR signaling can delay cell death and can repair damaged DNA. Overexpression of cyclin D1 was reported to have activated DDR signaling which was further activated by positive feedback through AKT/GSK3β/cyclin D1/Cdk4 pathway. The radioresistance of human tumor cells can be achieved via aberrantly activated DDR signaling

(Shimura *et al.*, 2010).

In spite of the possible correlation between aberrantly activated DDR signaling and radioresistance, it remains to be elucidated whether radioresistant cancer cells differentially modulate the expressions of DDR-related genes after ionizing radiation compared to radiosensitive cells. Additionally, because radiosensitive cancer cells also promptly attempt to arrest cell cycle progression, to recover damaged DNA, and/or to induce cell death after radiation, the temporal changes in DDR-related genes would be very important for understanding the underlying mechanisms of cellular response to radiation.

In this study, we used two non-small cell lung cancer (NSCLC) cell lines, which have different radiosensitivity (radiosensitive H460 and radioresistnat H1299). With these cell lines, we compared gene expression patterns of DDR-related genes after *in vitro* radiation. Further, the changes in the DDR-related gene expressions were confirmed via Western blot analysis. This result indicated that different radioresistance of H460 and H1299 might be affected by the different response of DDR signaling to radiation.

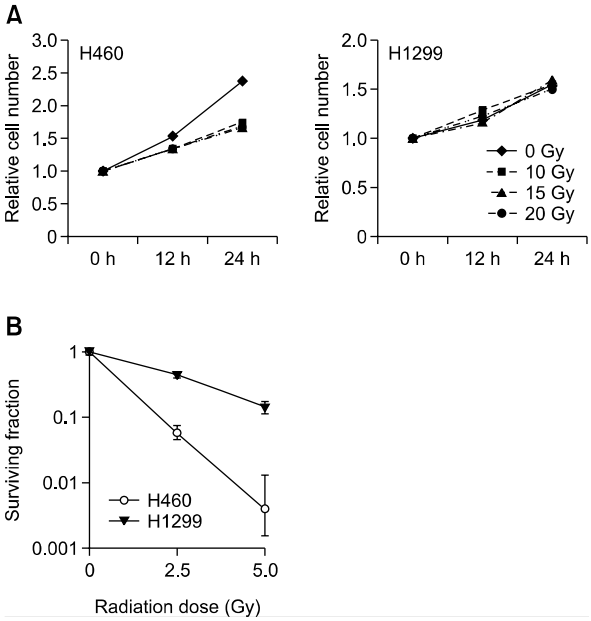


**Figure 1.** Radiosensitivity of NSCLC cell lines. The radiosensitivity of H460 and H1299 cells were compared via short-term survival and clonogenic assay. (A) For the short-term survival assay, the cells were irradiated with 10, 15, 20 Gy and harvested after 0, 12, 24 and 48 h. (B) The cells were treated with 2.5 and 5 Gy radiation, and the surviving fraction was calculated based on the number of colonies in the soft agar clonogenic assay.

## Results

### *In vitro* radio-resistance of NSCLC cell lines, H460 and H1299

As mentioned earlier, two kinds of NSCLC cell lines were used in this study, H460 (wild-type p53) and H1299 (p53-null) (Jeong *et al.*, 2009). Before comparing the expressional changes of the DDR-related genes, we first confirmed that they showed different radiosensitivity through short-term survival and clonogenic assay. In the short-term survival analysis, viability of H460 was significantly reduced 24 h after 10-, 15- and 20 Gy *in vitro* radiation (Figure 1A). In contrast, that of H1299 was not affected even when 20 Gy *in vitro* radiation was applied (Figure 1A). Concomitantly, the clonogenic potential of H460 was reduced by 2.5 and 5 Gy *in vitro* radiation significantly more than that of H1299 (Figure 1B), indicating that H1299 is more radioresistant than H460.

### Temporal changes in the expression of DNA damage checkpoint signaling genes in response to *in vitro* radiation

Although hundreds of genes are involved in the transduction of DDR signaling (Yoo *et al.*, 2004; Zhou and Bartek, 2004; Kumagai *et al.*, 2006), we select 24 DDR signaling genes that are primarily and directly associated with DDR signal transduction and that act as sensors, mediators, effectors and transducers (Supplemental Data Figure S1). To determine the differences in DDR signaling between radiosensitive H460 and radioresistant H1299 cell line, expressional changes of the 24 DDR signaling genes (Table 1) were compared via RNA expression arrays after 0, 2, 4, 8, 12 and 24 h of 10 Gy *in vitro* radiation. Generally, it is interesting that the temporal changes in several DDR signaling genes were clearly observed only in the radiosensitive H460 cell, while the expression level of the radioresistant H1299 cell was mostly unchanged after *in vitro* radiation (Figure 2A).

Further, we normalized each expression level to the average value of 0 h time point in both H460 and H1299, then, the genes were classified into four subgroups according to two criteria; (i) the difference in basal expression level between H460 and H1299 and (ii) the pattern of the temporal changes after *in vitro* radiation. In group A, the expression levels of MRE11A, ATM, ATR, p53BP1, CHK2, TOPBP1, CDC7, Claspin and CDC25C were relatively high and were generally unchanged in the radioresistant H1299 cells, but these genes were transiently decreased and restored after 4-6 h only in the radiosensitive H460 cells (Figures 2B and 3A). In other cases, NLRP2, SMC1B, MDC1 and CHK1 genes showed higher expression levels

**Table 1.** List of DNA damage response-related genes whose expressions were analyzed

| Genbank Acc. No. | Description |
|---|---|
| NM_005591 | MRE11 meiotic recombination 11 homolog A (S. cerevisiae) (MRE11A), transcript variant 1, mRNA. |
| NM_017852 | NLR family, pyrin domain containing 2 (NLRP2), mRNA. |
| NM_133482 | RAD50 homolog (S. cerevisiae) (RAD50), transcript variant 2, mRNA. |
| NM_000051 | Ataxia telangiectasia mutated (includes complementation groups A, C and D) (ATM), transcript variant 1, mRNA. |
| NM_133343 | RAD17 homolog (S. pombe) (RAD17), transcript variant 6, mRNA. |
| NM_133377 | RAD1 homolog (S. pombe) (RAD1), transcript variant 3, mRNA. |
| NM_004584 | RAD9 homolog A (S. pombe) (RAD9A), mRNA. |
| NM_004507 | HUS1 checkpoint homolog (S. pombe) (HUS1), mRNA. |
| NM_001184 | Ataxia telangiectasia and Rad3 related (ATR), mRNA. |
| NM_130384 | Three prime repair exonuclease 1 (TREX1), transcript variant 6, mRNA. |
| NM_022111 | Claspin homolog (Xenopuslaevis) (CLSPN), mRNA. |
| NM_014641 | Mediator of DNA damage checkpoint 1 (MDC1), mRNA. |
| NM_007027 | Topoisomerase (DNA) II binding protein 1 (TOPBP1), mRNA. |
| NM_005657 | Tumor protein p53 binding protein, 1 (TP53BP1), mRNA. |
| NM_007294 | Breast cancer 1, early onset (BRCA1), transcript variant BRCA1a, mRNA. |
| NM_001005735 | CHK2 checkpoint homolog (S. pombe) (CHEK2), transcript variant 3, mRNA. |
| NM_001274 | CHK1 checkpoint homolog (S. pombe) (CHEK1), mRNA. |
| NM_000546 | Tumor protein p53 (Li-Fraumeni syndrome) (TP53), mRNA. |
| NM_078467 | Cyclin-dependent kinase inhibitor 1A (p21, Cip1) (CDKN1A), transcript variant 2, mRNA. |
| NM_006306 | Structural maintenance of chromosomes 1A (SMC1A), mRNA. |
| NM_148674 | Structural maintenance of chromosomes 1B (SMC1B), mRNA. |
| NM_003503 | Cell division cycle 7 homolog (S. cerevisiae) (CDC7), mRNA. |
| NM_001789 | Cell division cycle 25 homolog A (S. pombe) (CDC25A), transcript variant 1, mRNA. |
| NM_001790 | Cell division cycle 25 homolog C (S. pombe) (CDC25C), transcript variant 1, mRNA. |

in the radioresistant H1299 cells than in the radiosensitive H460 cells and the expression patterns in both cell lines remained unchanged (group B; Figures 2B and 3B). On the contrary, p53, HUS1 and CDKN1A genes showed higher expression levels in radiosensitive H460 cells (group D; Figures 2B and 3D). Additionally, other gene sets such as TREX1, BRCA1, SMC1A, RAD17, RAD50, RAD1, RAD9A, and CDC25A were evenly expressed in both H460 and H1299 cells (group C; Figures 2B and 3C).

### Difference in the activation of DNA damage checkpoint signaling in response to *in vitro* radiation

To confirm the microarray results, we further investigated if the expression patterns of certain DDR signaling genes reflect the microarray analysis results. The p53 levels of H460 and H1299 were first addressed to validate the cell line models. As shown in Figure 4A, it is evident that p53 protein was highly expressed in the H460 cells, whereas it was barely detected in the H1299 cells. When the protein levels of several DDR-related genes were tested (Figure 4B), TopBP1 and Chk2, which were assigned to group A, were transiently reduced at 1 h and were recovered after 8 h following *in vitro* radiation treatments. Furthermore, one of the group B genes, Chk1, was shown to be constitutively higher in the radioresistant H1299 cells, validating the microarray data and pattern classification. Interestingly, it is notable that DDR signaling was strengthened in the radioresistant H1299 cells. For example, the phosphorylation levels of Chk1 and Chk2 in H1299 were stronger than those of H460 and were slightly prolonged. Taken together, the temporal changes in the DDR-signaling genes such as TopBP1, Chk1 and Chk2 supported the microarray results. It was also found that both H460 and H1299 rapidly activated DDR signaling in response to *in vitro* radiation but DDR signaling seems to be higher in the H1299 cells.

## Discussion

In this study, two NSCLC cell lines were applied to explore radiosensitivity as well as time-dependent alteration in the gene expression profile after *in vitro* radiation. H460 cells were found to be relatively sensitive to radiation, showing a decreased cellular proliferation rate and clonogenic potential, whereas the H1299 cells seemed to be radioresistant (Figure 1). When the gene expression patterns of H460 and H1299 were compared, it is of interest that only H460 changed the expressions of the

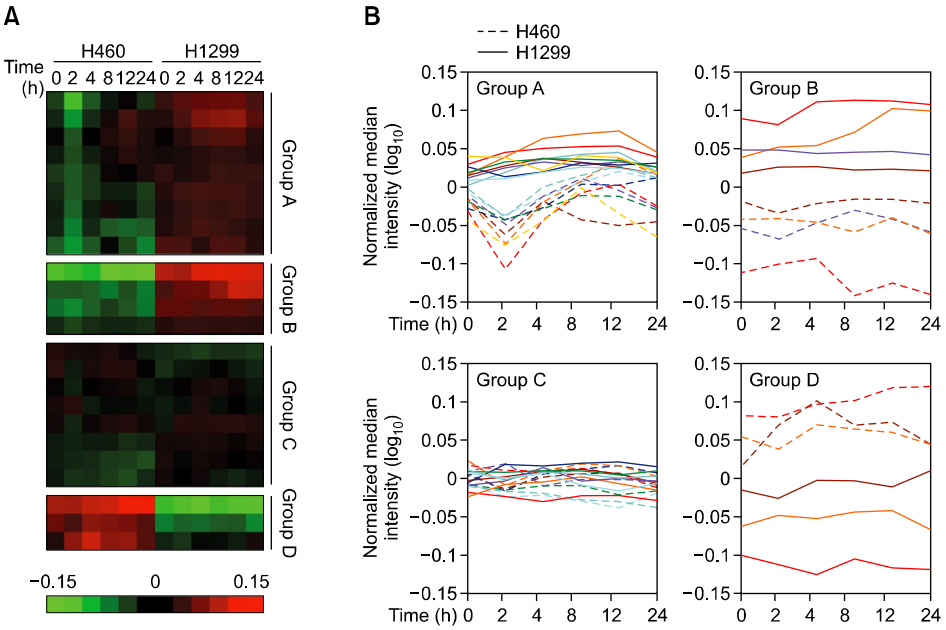


**Figure 2.** Expressional alteration patterns of the DNA damage checkpoint signaling genes in response to *in vitro* radiation. The expressional changes of the 24 DNA damage checkpoint signaling genes were analyzed via microarray expression analysis 0, 2, 4, 8, 12 and 24 h after 10 Gy radiation and were categorized into four subgroups according to the temporal expression patterns. Each level was normalized to the average value of both the H460 and H1299 controls and was visualized with ClusterTM /Treeview analysis program (A) or log-scale graph (B).

DDR-related genes after *in vitro* radiation, and that H1299 did not (Figures 2 and 3). Regarding the fact that the temporal regulation of the target genes might be critical for deciding whether to resume cell proliferation, to enter senescence, or to induce apoptosis (Snyder and Morgan, 2004), the transient suppression of several DDR-related genes in H460 might be important for modulating DDR signaling and radiosensitivity.

Although H1299 is relatively radiosensitive, some cells ultimately survived and made visible colonies in the clonogenic assay (Figure 1). Many previous studies have reported that radioresistant subclones could be artificially derived from radiosensitive cancer cells (Tyrsina *et al.*, 2005; Ogawa *et al.*, 2006; Nojiri *et al.*, 2009). According to our observation, it is likely that most of the genes in group A was associated with the initial step of signal transduction, which contributed as signal sensors and transducers (ATM, ATR, MRE11, 53BP1, TopBP1, Claspin and Chk2). This can be explained by the fact that the radiosensitive H460 cells temporally undergo DDR signaling attenuation (group A, within 2 h), resulting in the impairment of the proper response, such as the DNA repair process. Rather, they promptly respond to radiation by suppressing cell proliferation through the induction of cell cycle arrest genes in group D (p53 and CDKN1A). This might initially lead to the cell cycle arrest and cell death of radiosensitive H460 cells. After several hours, these cells actively restored the expressions of the group A genes to sustain intact DDR signaling, which might have been responsible for the recovery from the radiation-induced DNA damages and acquired radioresistance. This provides a clue for targeting DDR signaling and for developing anti-cancer therapeutics based on the underlying mechanisms in response to radiation, but much remains to be elucidated.

It has been well-known that H460 harbors wild-type p53, whereas H1299 does not express p53 (Choi *et al.*, 2000; Kataoka *et al.*, 2000; Lai *et al.*, 2000). In H460 cells, it is evident that ionizing radiation normally activates p53 and induces p21 mRNA expression through DDR signaling (Figures 3D and 4A). This tendency was consistently observed in other studies (Amundson *et al.*, 1999; Tusher *et al.*, 2001; Sak *et al.*, 2003). Conversely, although H1299 cells can initiate DDR signaling at the level of phospho-Chk1/2 (Figure 4B), the absence of master regulator p53 enables H1299 cells to ignore the radiation-induced cell cycle arrest and the subsequent apoptosis (Figures 1, 2 and 4A). It has been also observed that p53-null H1299 cells are more resistant to anti-tumor therapy than H460 cells (Nishizaki *et al.*, 2001). Similarly, the restoration of p53 signaling through the ectopic expression of wild-type p53 or MDM2-targeting antisense

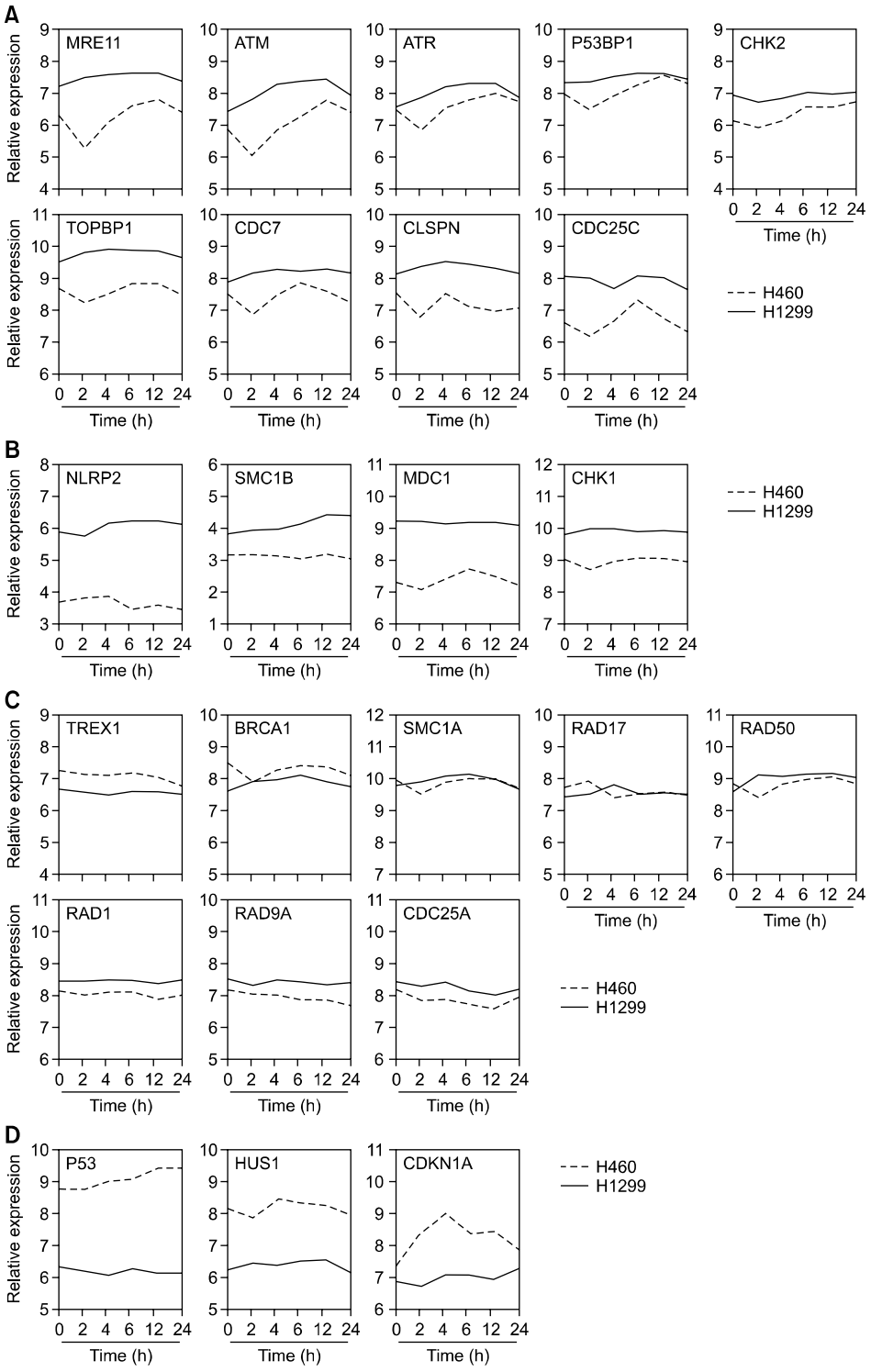


**Figure 3.** Changes in the DDR-related genes after *in vitro* radiation. According to our classification, the time-course changes in each gene in H460 and H1299 were plotted and visualized [(A), group A; (B), group B; (C), group C; (D), group D].

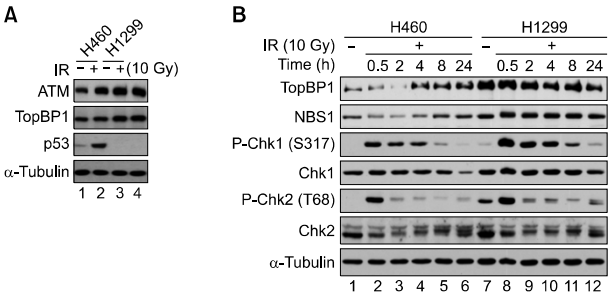


**Figure 4.** Differential activation of DDR signaling after *in vitro* radiation. The activation of DDR signaling was analyzed via Western blotting. (A) The protein levels of certain DDR-related genes in H460 and H1299 were addressed after *in vitro* radiation (10 Gy). (B) The H460 and H1299 cells were treated with *in vitro* radiation (10 Gy) and were harvested after 0.5, 2, 4, 8, and 24 h. Then, the temporal changes in the DDR-related genes such as TopBP1, NBS1, Chk1 and Chk2 were visualized via Western blotting.

oligonucleotide causes human cancer cells to become radiosensitive (Zhang *et al.*, 2004; Mazzatti *et al.*, 2005), suggesting that the lack of p53 function allows cell proliferation rather than apoptosis-mediated elimination. Thus, it is plausible that a different p53 status may be primarily devoted to the radiosensitivity of H460 and H1299. However, it has to be elucidated how subpopulation of p53-expressing cancer cells such as H460 could acquire radioresistance after *in vitro* radiation.

It is also worthwhile to investigate the direct relationship between p53 and different DDR-related gene expressions. As the temporal reduction of the group A genes was restrictively observed in the H460 cells, p53 possibly contributes to the expressions of DDR-related genes. Indeed, several reports have demonstrated that p53-mediated transcriptional suppression affects the expression of several DNA repair and cell survival genes, such as survivin, deoxyuridine triphosphate nucleotidohydrolase (dUTPase), manganese superoxide dismutase (Mn-SOD), apurinic/apyrimidinic (AP) endonuclease and myeloid cell leukemia sequence 1 (MCL-1) (Mirza *et al.*, 2002; Lohr *et al.*, 2003; Dhar *et al.*, 2006; Zaky *et al.*, 2008; Wilson *et al.*, 2009). Thus, it is possible that the temporal reduction of the group A genes is caused by radiation-induced p53 activation.

In conclusion, the present study on the temporal changes in the DDR-related gene expressions after *in vitro* radiation provides a novel insight for the short-term response of NSCLC cell lines. By comparing two NSCLC cell lines that have different radiosensitivity levels and p53 statuses, it was revealed that the transient reduction of certain DDR genes can be observed only in radiosensitive H460 cells, which was confirmed via Western blotting. Furthermore, the possibility that p53 is involved in these events was discussed. Collectively, this serves as a basis for the transcriptional control of DDR-related genes, which highlights the first step to uncovering the molecular responses of lung cancer cells to radiation.

## Methods

### Cell culture

H460 and H1299 cells were maintained in Roswell Park Memorial Institute medium 1640 (RPMI1640) supplemented with 10% FBS (Hyclone, Logan, UT), 1% penicillin/streptomycin (Gibco-BRL, Carlsbad, CA), and 2 mM L-glutamine (Gibco-BRL, Carlsbad, CA).

### Survival and clonogenic assay

In the survival assay, H460 or H1299 cells in 96-well plates were irradiated (0, 10, 15 and 20 Gy) using a blood irradiator (IBL-437C, CIS-US, Inc., Bedford, MA). The cell viability was determined via colorimetric assay (Cell Counting Kit-8, Dojindo Molecular Technologies, Gaithersburg, MD) 0, 12, 24 and 48 h after radiation (n = 3 for each group).

For the clonogenic assay, the cells were seeded into 6-well plates at a density of 200-500 cells/well and were incubated overnight. After exposure to ionizing radiation from a blood irradiator (0, 2.5 and 5 Gy), the cells were cultured for 10-15 days. The colonies were fixed with 100% chilled methanol and were stained with crystal violet (Sigma, St. Louis, MO). The colonies with over 50 cells were scored and the cell survival was determined after correcting for plating efficiency.

### RNA expression array and data analysis

Total RNAs were isolated from 10 Gy irradiated H460 or H1299 cells at 0, 2, 4, 8, 12, and 24 h after radiation (n = 3 for each group) using TRIZOL reagent (Invitrogen, Carlsbad, CA) and were further purified using RNeasy Mini Kit (Qiagen, Hilden, Germany). The samples were processed and hybridized to Affymetrix U-133 plus2 GeneChip Arrays according to the Affymetrix GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). All the arrays were confirmed to be within the acceptable minimal quality control parameters. The gene expression CEL files were normalized using the Robust Multichip Averaging procedure and PM-MM difference model was used to obtain the expression values. Statistical analysis comparing the expression of the 24 DNA damage checkpoint signaling genes was performed using R Package Software 2.6.0. The normalized value was visualized by using the Cluster™ and Treeview programs according to the developer's instructions (Eisen Lab, http://www.eisenlab.org).

### Western blot analysis

The cells were lysed and prepared for immunoblotting analysis as previously described (Yoo *et al.*, 2009). The cells were processed at the indicated time after irradiation (10 Gy), were washed (PBS), and were harvested. Anti-ATM, anti-Chk1, anti-Chk2, anti-Rad17, anti-p21, and anti-

p53 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Chk1 phospho-Ser317 and anti-Chk2 phospho-Thr68 antibodies were obtained from Cell Signaling Technology (Danvers, MA). Anti-TopBP1, anti-Nbs1, and anti-human $\alpha$-tubulin antibodies were obtained from Bethyl Laboratoratoies, Inc (Montgomery, TX), Calbiochem (La Jolla, CA) and Oncogene Research Products (Cambridge, MA), respectively.

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### Supplemental data

Supplemental data include a figure and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-43-7-06.pdf.

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