Effect of PP2A on p34^{SEI-1} expression in response to ionizing radiation in MCF-7 human breast cancer cells

SAMIL JUNG[1], HYEON-KYUNG JEONG[1], JIN SHIN[2] and MYEONG-SOK LEE[1]

[1]Research Center for Women's Diseases, Division of Biological Sciences, Sookmyung Women's University, Seoul 140-742; [2]Songdo Technopark, Inchon 406-840, Republic of Korea

Received November 15, 2010; Accepted January 27, 2011

DOI: 10.3892/ijo.2011.950

Abstract. Breast cancer is one of the most common cancers in women and it is highly treatable by radiotherapy and/or radiochemotherapy. A global analysis of the protein expression pattern was performed to identify radiation-responsive proteins in MCF-7 breast cancer cells using 2D-PAGE coupled with MALDI-TOF-MS. When MCF-7 cells were exposed to ionizing radiation (IR) such as γ-rays, eight proteins (GH2, RGS17, BAK1, CCNH, TSG6, RAD51B, IGFBP1, and CASP14) were up-regulated and three proteins (C1QRF, PLSCR2, and p34^{SEI-1}) were down-regulated. In an effort to find what mechanisms are responsible for these changes, we initially focused on p34^{SEI-1}, which is known as a transcriptional regulator and oncogene. Our results show that p34^{SEI-1} expression is significantly decreased only at the protein level but not at the transcriptional level after IR treatment. We suggest that the B55 regulatory subunit of PP2A, a positive regulator of p34^{SEI-1}, is at least partly responsible for the decreased p34^{SEI-1} expression, in which the B55 regulatory subunit of PP2A was down-regulated at the protein level as a cellular response to IR. We, therefore, propose that inactivated PP2A resulting from the absence of the B55 subunit may not be able to dephosphorylate p34^{SEI-1} and therefore increase the phosphorylated form of p34^{SEI-1} with low stability. Our further extended study shows that the p34^{SEI-1} expression level was not changed after H_2O_2 treatment at either protein or transcriptional levels. This result implies that MCF-7 cells seem to use different signaling pathways in response to IR and H_2O_2 stresses although both of them belong to the same DNA damage inducing stimuli of reactive oxygen species (ROS).

Correspondence to: Dr Myeong-Sok Lee, Division of Biological Science and Research Center for Women's Diseases, Sookmyung Women's University, Seoul 140-742, Republic of Korea
E-mail: mslee@sookmyung.ac.kr

Abbreviations: IR, ionizing radiation; p34^{SEI-1}, cycline-dependent kinase 4 (cdk4)-binding protein; PP2A-B55, protein phosphatase type 2A containing B55 regulatory unit

Key words: ionizing radiation, p34^{SEI-1}, PP2A-B55

Introduction

Breast cancer is a common malignancy worldwide and is a leading cause of death in women over the world. Radiotherapy is generally used in conjunction with chemotherapy, hormone-therapy, or surgery. Radiotherapy is considered as an important treatment of cancer as it can be used to treat most common types of cancer to some extent. Radiation is known to activate multiple signaling pathways for cancer cells to be inactivated, resulting in diverse stress responses including apoptosis, cell cycle arrest, senescence, and gene induction. Many aspects of the initial response to radiation-induced DNA damage have been extensively analyzed via p53 and other DNA damage checkpoint responses (1,2). Although several mechanisms are known to be involved in these processes, the molecular mechanisms inactivating tumor cells in response to radiation is not yet fully elucidated. Moreover, many tumors fail to respond to radiotherapy because the tumors have become less sensitive and more resistant to it after constitutive treatments. Although many studies on the molecular mechanisms of resistance to radiotherapy have been carried out, problems related to overcoming this resistance remains to be solved. Therefore, it will be helpful to identify radiation-responsive genes to better understand the molecular mechanisms involved in the response of tumors to radiation and ultimately to improve radiotherapy. Many radiation-responsive genes have been identified through different approaches. Among them, stress-responsive effector genes are known to be inducible by radiation, and their products participate in the radiation induced response (3-5). The major radiation-responsive effector genes include *RAF1*, *p21*, *p53*, *GADD45A* (*GADD45*), 14-3-3 sigma, *BAX, TNFRSF (Fas/APO1), TNFRSF 10B (KILLER/DR5), PIG3, THBS1 (TSP1), IGFBP3, and DIR1 (6-9).* They play key roles in the cellular response to radiation-induced stress by modulating cell cycle checkpoints, apoptosis, and DNA repair (3-4,10-13). Kis *et al* identified another set of radiation-responsive genes using microarray analysis in primary human fibroblasts (14). They detected about 200 IR responsive genes at the transcriptional level, in which 30 genes (28 up- and 2 down-regulated) responded to radiation in all investigated cells and 20 were functionally grouped. They belong to one of the following groups: the DNA damage response (GADD45A, BTG2, PCNA, and IER5), regulation of cell cycle and cell proliferation (CDKN1A, PPM1D, SERTAD1, PLK2, PLK3,

and CYR61), programmed cell death (BBC3 and TP53INP1), signaling pathways (SH2D2A, SLIC1, GDF15, and THSD1), and other functions (SEL10, FDXR, CYP26B1, and OR11A1) (14). However, the mRNA expression profiles did not match up to their protein expression profiles because radiation-responsive genes seem to respond differently at the transcriptional and the protein levels.

The objective of the present study was originally aimed to identify the radiation-responsive genes at the protein levels in MCF-7 human breast cancer cells using the 2D-PAGE and MALDI-TOF-MS. In this process, we observed that the expression level of p34^{SEI-1} oncogene, one of the differently expressed proteins, was down-regulated at the protein level by the inhibition of PP2A phosphatase enzyme activity after being exposed to IR.

Materials and methods

Condition of MCF-7 cell culture and treatment of IR, H_2O_2, and okadaic acid (OA). The MCF-7 human breast cancer cell line was purchased from the American Type Culture Collection (ATCC, USA). MCF-7 cells were cultured at 37˚C in a humidified atmosphere composed of 95% air and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) media (WelGENE, Korea). This medium was supplemented with 10% fetal bovine serum (Gibco-BRL, Korea) and 1% antibiotic-antimycotic (Gibco-BRL). To induce an IR response, the MCF-7 cells were irradiated with γ-rays with a ^{137}Cs γ-ray source (Atomic Energy of Canada, Ltd., Ontario, Canada) and harvested after indicated times of incubation at 37˚C for further experiment. The MCF-7 cells were also cultured in a medium containing different concentrations of H_2O_2 (Sigma, Korea) as indicated. To inhibit the enzyme activity of PP2A protein phosphatase, the MCF-7 cells were treated with 0.1 mM of OA, which was purchased from LC Labs (Cat No. O-2220, USA) and dissolved in dimethyl sulfoxide (DMSO, Sigma, Korea).

Protein extraction. Cells were harvested and washed twice with cold PBS (phosphate-buffered saline) buffer and centrifuged at 3,000 rpm, 4˚C for 3 min. The supernatant was removed and then the cells were resuspended with lysis buffer [500 mM HEPES (pH 8.5), 4% CHAPS, 8 M urea, 1 μg /ml aprotinin, 100 μg/ml PMSF, and 2.4 mg/ml DTT], sonicated for 10 sec on the ice, centrifuged at 13,000 rpm, 4˚C for 10 min, and then harvested.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), gel scanning, and image analysis. The first dimensional isoelectric focusing (IEF) was performed on precast 18 cm immobilized pH 3-10 gradient (IPG) strips (Amersham Pharmacia Biotech) at 20˚C using a commercial flatbed electrophoresis system (IPGphor; Amersham Pharmacia Biotech). The total proteins of 500 μg were mixed with a rehydration buffer containing 8 M urea, 2% (w/v) CHAPS, 2.4 mg/ml DTT, 2% (v/v) IPG buffer, and a trace of bromophenol blue for the tracking dye. The mixtures were loaded onto the IPG strip, followed by 12 h of active rehydration at 50 V, 50 μA and ramped to 500 V, 50 μA over a period of 10 min and then kept at 5,000 V, 50 μA for 1 h. At the end of the first dimension run (80 kV·h), the IPG strips were immediately loaded onto 11% SDS-PAGE gels and held in place with 0.5% agarose dissolved in a SDS-PAGE running buffer. In the second dimension, the SDS-PAGE was performed for separation without a stacking gel at 150 V, 20 mA for about 20 h per gel in an SDS-electrophoresis buffer (25 mM Tris, 92 mM glycine, and 0.1% SDS). After the electrophoresis separation, the gels were stained by silver-staining. The stained 2-D gels were scanned on a Las-3000 (Fuji Photo Film Co.) by using the 2-D software PDQuest (Bio-Rad). The different gel patterns were then automatically matched to each other and the quantities of matched spots in different gels were compared. The molecular masses and pI were calculated by the PDQuest software using the selected pI and Mr standard proteins.

Matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS). Proteins from gels were identified by mass spectrometry (MALDI-TOF). After tryptic in-gel digestion of 2D-PAGE resolved proteins, samples for MALDI peptide mass mapping were prepared as described previously (15). Mass spectra were obtained using Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems, Cambridge, MA). Proteins were identified according to their tryptic peptide mass fingerprint after database searching performed the MS-Fit, which is accessible over World Wide Web at http://prospector.ucsf.edu/. MS-Fit performed fast database search by comparing experimentally determined masses from proteolytic digestion of protein with peptide database masses calculated from NCBInr protein database and SWISS-PROT accession numbers.

Cell cycle analysis by fluorescence-activated cell sorting (FACS). For FACS analysis, cells were harvested at the indicated time points, washed twice in ice-cold PBS buffer, and fixed by resuspending them in absolute ethanol for 30 min. The fixed cells were centrifuged at 1,500 rpm for 5 min and washed twice with cold PBS buffer. The cell pellets were resuspended in 0.5 ml PBS containing 50 μg/ml propidium iodide (Sigma-Aldrich Chemical Co., Korea), 10% sodium citrate (Sigma), 100 μg/ml RNase (Invitrogen, Korea), and 0.001% NP40 (Sigma). Following the incubation at 37˚C for 30 min in the dark, the samples were analyzed by a FACScan flow cytometer (Becton-Dickinson FACScan, Sunnyvale, CA, USA) equipped with CellQuest 3.2 software (Becton-Dickinson).

Western blot analysis. The cells were centrifuged at 3,000 rpm for 3 min and washed twice in an ice-cold PBS buffer. The cells were then lysed in EBC lysis buffer and the protein amount was quantified using a protein assay kit (Bio-Rad). Approximately 15 μg of total protein per sample was subjected to SDS-PAGE and transferred to an Immobilon polyvinylidene difluoride filter (Millipore). The filter was blocked in 5% non-fat dry milk/0.1% Tween/TBS followed by incubation with each corresponding antibody and immunodetection was done using the ECL System (Amersham Pharmacia Biotech). Antibodies used in this study were purchased as follows: p34^{SEI-1} (Enzo Life Sciences, ALX-804-645), PP2A-B55 (Santa Cruz, sc-18330), p-Chk1(Ser345) (Cell Signaling, no. 2341), and γ-tubulin (Santa Cruz, sc-7396).

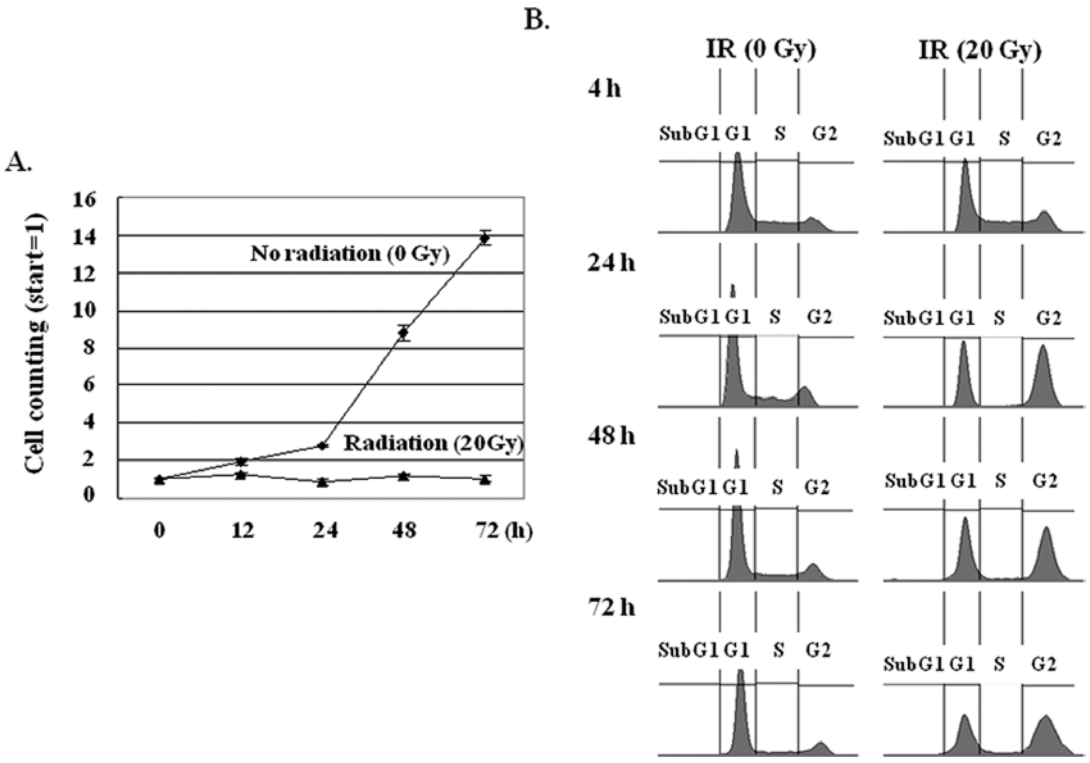

Figure 1. IR-induced cell cycle arrest at the G2 phase in MCF-7 cells. (A) Cell growth analysis of MCF-7 cells in response to IR. MCF-7 cells were exposed to 20 Gy of IR, incubated for the indicated times, and then the growth rate was checked. (B) FACS analysis of MCF-7 cells either untreated or treated with 20 Gy of IR. One representative experiment of three is shown.

Reverse transcription (RT)-PCR. The total RNA was extracted from MCF-7 cells using the RNeasy Minikit (Qiagen). For reverse transcription, one μg RNA of each sample was subjected to cDNA synthesis using an oligo (dT) primer and the Revert Aid First Strand cDNA Synthesis kit (Fermentas, Korea) following the manufacturer's instructions. PCR amplification was performed using 10 ng cDNA, different sets of primers, and AccuPower PCR PreMix (Bioneer, Korea). The 142-bp of p34^{SEI-1} gene products were amplified with p34^{SEI-1}-RT-F (5'-ACATTGAGGGCCTGAGTCAG-3') and p34^{SEI-1}-RT-R (5'-TCAAGCCCATCGTCCAGTAG-3') primers at 72˚C for 27 cycles. As an internal control, the 377-bp of β-actin gene products were generated using pACTB-RT-F (AGGTCGGAG TCAACGGATTTG) and pACTB-RT-R (GTGATGGCATG GACTGTGGT) primers at 58˚C for 21 cycles. The amplification reaction was carried out using the GeneAmp PCR System 9700 from Applied Biosystems. The amplification products were electrophoresised on a 2% agarose gel stained with ethidium bromide and visualized in a LAS-3000 imaging system.

Results

G2 cell cycle arrest induced by IR in MCF-7 cells. We initially exposed the MCF-7 breast cancer cells to various γ-ray doses of 1, 5, 10, or 20 Gy. Cell growth was repressed in the 20 Gy radiation treated cells as shown in Fig. 1A. IR treatment of the MCF-7 cells did not affect cell viability as assessed by the trypan blue exclusion test (data not shown). Instead, the cell growth was repressed due to cell cycle arrest at the G2 phase as revealed in FACS analysis (Fig. 1B). This result implies that IR irradiated MCF-7 cells undergo cell cycle arrest rather than apoptosis. The data are consistent with a previous study that IR induces cell cycle arrest but fails to activate the mitochondrial death pathway in MCF-7 cells (16). Then, our curiosity about what genes are responsible for this phenotype prompted us to identify the radiation-responsive genes in MCF-7 cells.

Identification of p34^{SEI-1} as a radiation-responsive gene in MCF-7 cells. To identify radiation-responsive genes, MCF-7 breast cancer cells were irradiated with 20 Gy of γ-rays and harvested after incubation for 12, 24, and 48 h. We then observed differentially expressed proteins using 2D-PAGE and MALDI-TOF-MS as described in Materials and methods. Proteins of about 1,000 to 1,200 spots in each gel were visualized by silver staining and detected by PDQuest 2D-image-analysis software (Bio-Rad). After spot detection, the gels were matched to each other with the help of the so-called landmark function and about 730 spots in total were matched in all gels analyzed according to PDQuest software. For internal standards, 9 spots were chosen randomly to calculate the deviation of the spot position and identified by MALDI-TOF-MS. These identified proteins include LMNA, PPP3CA, HNRPH3, CA13, BTEB1, SCN3B, ZFX, PPIA, and C20ORF179. They were used for the generation of a relevant pI and Mr scale for the whole pattern. When the expression patterns of proteins were compared with the control gel, many spots were found to differ greatly. Among them, 11 spots were found to be significantly different. One set of proteins were up-regulated in response to IR in MCF-7 cells. They include GH2, RGS17, BAK1, CCNH, TSG6, RAD51B, IGFBP1, and CASP14. More

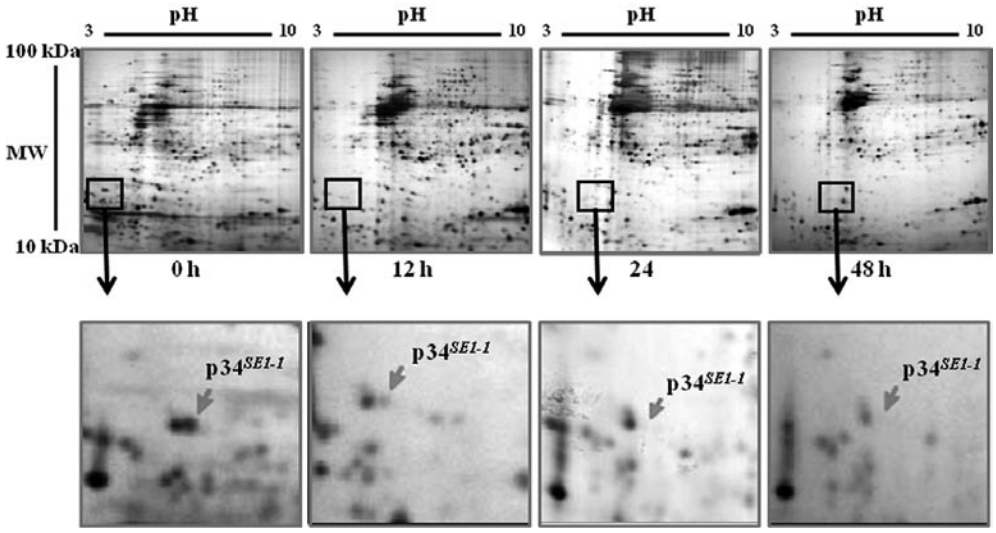

Figure 2. Down-regulated p34^{SE1-1} protein expression after exposure to IR. MCF-7 cells were treated with 20 Gy of IR and incubated for indicated times. The horizontal axis is the isoelectric focusing dimension, which stretches from pH 3 (left) to pH 10 (right). The vertical axis is the polyacrylamide gel dimension, which stretches from 10 kDa (bottom) to 100 kDa (top). In the upper panels, protein expression patterns are shown from the left of top. The regions containing p34^{SE1-1} protein are indicated with the black rectangles. In the lower panels, p34^{SE1-1} protein is indicated with the arrows on the enlarged gels, which shows the difference more clearly.

Table I. Differentially expressed proteins in response to IR in MCF-7 cells.

	An[a]	Gene	Protein	pI/Mw (Da)[b]
Class I[c]	O15315	RAD51B	DNA repair protein RAD51 homolog 2 (R51H2)	5.8/38257
	P51946	CCNH	Cyclin H	6.7/37644
	P98066	TSG6	Tumor necrosis factor-inducible protein TSG-6	6.5/31232
	P01242	GH2	Growth hormone variant	7.6/25000
	Q9UGC6	RGS17	Regulatior of G-protein signaling 17	5.6/24360
	Q16611	BAK1	Bcl-2 homologous antagonist/killer	5.7/23409
	P08833	IGFBP1	Insulin-like growth factor binding protein 1	5.1/27904
	P31944	CASP14	Caspase-14	5.4/27680
Class II[d]	O75973	C1QRF	C1q-related factor	5.3/26453
	Q9NRY7	PLSCR2	Phospholipid scramblase 2	5.5/25523
	Q9UHV2	p34^{SE1-1}	Cycline-dependent kinase 4 (cdk4)-binding protein	4.3/24674

[a]AN, SWISS-PROT accession number from the SWISS-PROT database. [b]pI and molecular weights were calculated by the PDQuest software utilizing the selected pI and Mr standard proteins. [c]Increased protein expression in response to IR in MCF-7 cells. [d]Decreased protein expression in response to IR in MCF-7 cells.

information is listed in Table I. Most of these proteins are known to be involved in the cellular processes of cell cycle control, apoptosis, DNA repair, cell proliferation, and other functions. In another set, the expression levels of three proteins were down-regulated after the exposure of γ-rays. They include C1QRF, PLSCR2, and p34^{SE1-1}. p34^{SE1-1}, the subject of this report, encodes a cycline-dependent kinase 4 (cdk4)-binding protein. It is identical to the TRIP-Br1 (transcriptional regulator interacting with the PHD-bromodomain 1) and SERTAD1 (SERTA domain containing 1). A considerable amount of p34^{SE1-1} was detected in the control gel (0 h). However, the expression level of p34^{SE1-1} started to decrease from 12 h and significantly down-regulated after 48 h (Fig. 2). The regions containing p34^{SE1-1} are marked by black rectangles and magnified on the lower gels. It is known that p34^{SE1-1} functions in both the transcriptional regulation and cell cycle progression working with the E2F1/DP1 transcription complex. It positively regulates the cell cycle progression by rendering the activity of Cyclin D/CDK4 (Cyclin dependent kinase 4) resistant to the inhibitory effects of p16^{INK4a} after forming a stable quaternary complex with Cyclin D, CDK4, and p16^{INK4a} (17-20). In addition, the p34^{SE1-1} functions as an onco-protein in cells as

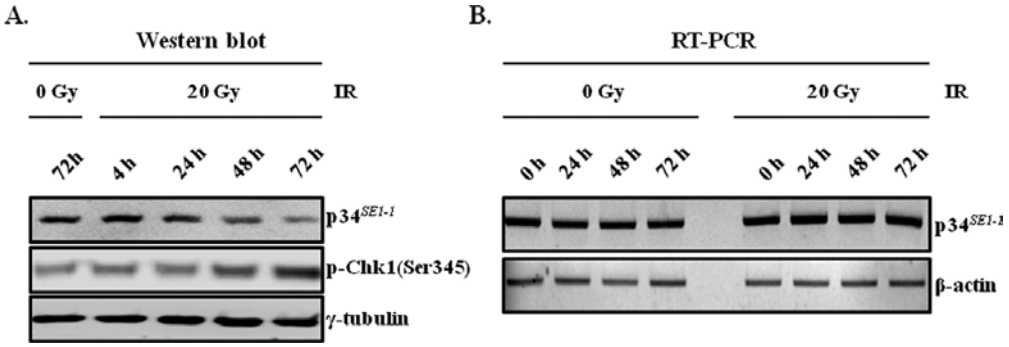

Figure 3. Western blot and RT-PCR analyses of p34^{SEI-1} expression after exposure to IR. MCF-7 cells were exposed to 20 Gy of γ-rays and incubated for the indicated times in a growth medium. (A) Western blot analysis of p34^{SEI-1} expression at the protein level in MCF-7 cells. p-Chk1(Ser345) was used as a positive control for IR-induced cell cycle arrest. (B) RT-PCR analysis of p34^{SEI-1} expression at the transcriptional level. As loading controls, γ-tubulin and β-actin were used for Western blot and RT-PCR analyses, respectively. One representative experiment of three is shown.

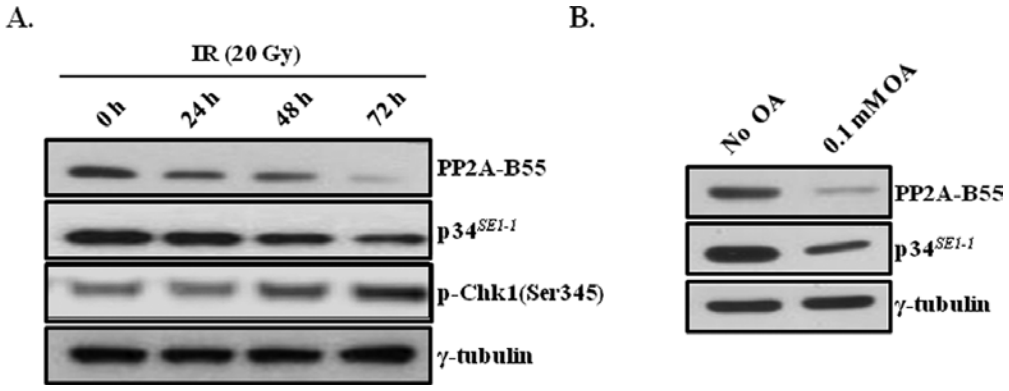

Figure 4. Effect of PP2A-B55 on p34^{SEI-1} expression in response to IR. (A) The levels of PP2A-B55 and p34^{SEI-1} expression after IR exposure. MCF-7 cells were exposed to 20 Gy of γ-rays and incubated for the indicated times in a growth medium. p-Chk1(Ser345) was used as a positive control for IR-induced cell cycle arrest. (B) Effect of OA on PP2A-B55 and p34^{SEI-1} expression. The levels of PP2A-B55 and p34^{SEI-1} proteins were measured from cells grown in the presence or absence of 0.1 mM of OA. γ-tubulin was used as loading control in Western blot analysis. One representative experiment of three is shown.

shown in recent studies. For example, p34^{SEI-1} is able to induce oncogenic transformation of mouse fibroblasts (21). The p34^{SEI-1} gene is also found to be highly expressed in carcinomas from pancreatic, lung, and ovarian tissues (17,22,23). Our previous study showed that p34^{SEI-1} expression is absent or weak in normal tissues but is strongly expressed in tissues obtained from patients with breast cancer, in which the anti-apoptotic function of p34^{SEI-1} increases tumor cell survival by protecting the X-linked inhibitor of apoptosis protein (XIAP) from degradation (24).

In addition to these well known facts about p34^{SEI-1}, our data imply that the p34^{SEI-1} protein may also be involved in the IR induced tumor cell inactivation. In other words, IR leads to the loss of the oncogenic function of p34^{SEI-1} through a decrease in the p34^{SEI-1} protein level and inactivates tumor cells.

Decreased p34^{SEI-1} expression at the protein level but not at the transcriptional level. Our 2D-PAGE result suggests that IR exerts a negative effect on the p34^{SEI-1} expression at the protein level. This was further tested using a Western blot analysis. MCF-7 cancer cells were irradiated with 20 Gy of γ-rays and then harvested after incubation for 4, 24, 48, and 72 h. The level of p34^{SEI-1} protein was significantly decreased after 48 h compared to IR non-treated cells (Fig. 3A). Radiation-induced phosphorylation of Chk1 at Ser345 causes p53-dependent cell cycle arrest at the G2 phase (25). Therefore, phosphorylated Chk1 at Ser345 [p-Chk1(Ser345)] was used as a positive control. Discovery of the inverse relationship between γ-ray treatment and the p34^{SEI-1} protein level suggested the possibility of the same situation at the transcriptional level. To test the hypothesis, the total RNA was isolated from the MCF-7 cells after the exposure to IR as indicated in Materials and methods. The transcriptional level of the p34^{SEI-1} gene was measured using RT-PCR. Our result shows that radiation-induced transcriptional down-regulation was not detected in the p34^{SEI-1} gene expression (Fig. 3B). Taken together, IR affects p34^{SEI-1} expression only at the protein level not at the transcriptional level.

Effect of IR-induced PP2A inactivation on p34^{SEI-1} expression. The next question was what mechanism is responsible for the decreased p34^{SEI-1} expression after exposure to IR. Zang *et al* recently suggested that inhibition of PP2A activity increases the accumulation of phosphorylated p34^{SEI-1} (p34^{SEI-1}-SerP)

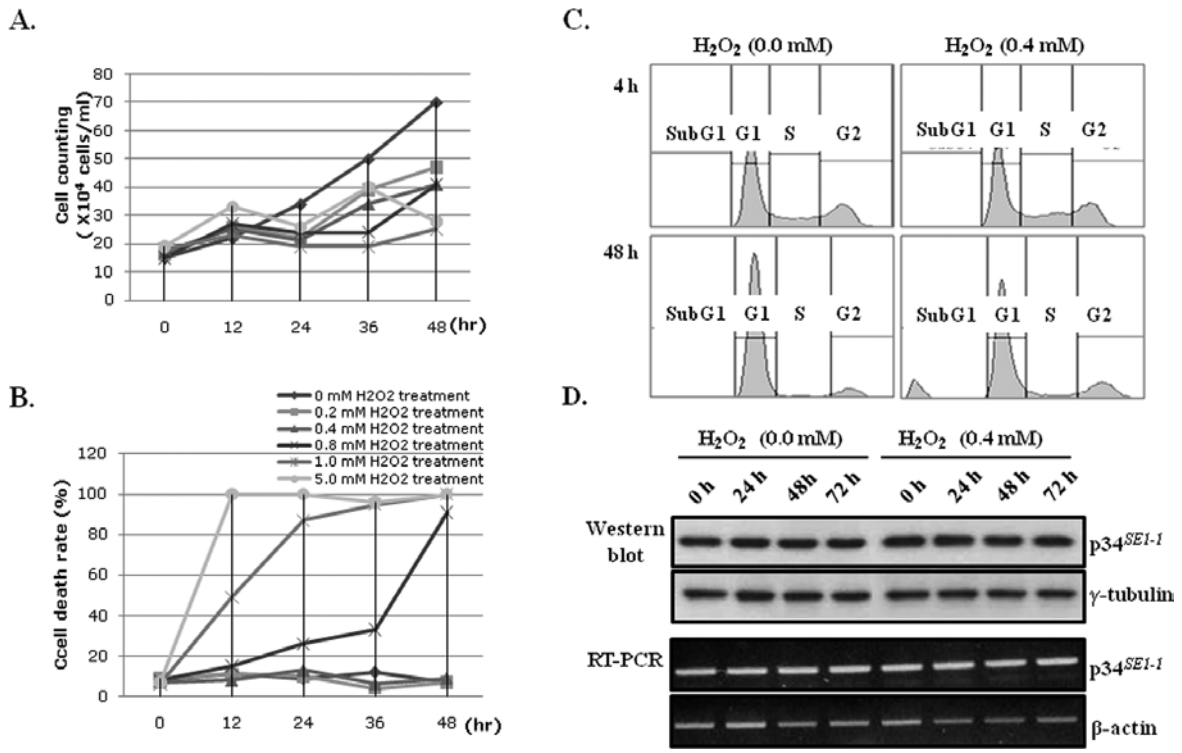

Figure 5. Effect of H_2O_2 on p34^{SEI-1} expression in MCF-7 cells. (A) MCF-7 cells were treated with different concentrations of H_2O_2 for the indicated times and cell growth was checked. (B) Cell death rate of MCF-7 cells after treatment with different concentration of H_2O_2. Cell death was assessed by trypan blue uptake. (C) FACS analysis of MCF-7 cells either left untreated (control) or treated with 0.4 mM of H_2O_2. The various cell cycle phases are indicated. One representative experiment of three is shown. (D) Western blot and RT-PCR analyses of p34^{SEI-1} expression in MCF-7 cells. As loading controls, γ-tubulin and β-actin were used for Western blot and RT-PCR analyses, respectively. One representative experiment of three is shown.

with low stability and finally decreases the total amount of p34^{SEI-1} protein (26). PP2A-ABαC interacts with p34^{SEI-1} and dephosphorylates p34^{SEI-1} in the cytoplasm (26). In eukaryotic cells, PP2A is an essential serine/threonine phosphatase that regulates a variety of key steps in metabolism, replication, transcription, translation, metabolism, cell cycle progression, cell division, and apoptosis (27-29). PP2A consists of a 65-kDa scaffolding subunit (A), a 36-kDa catalytic subunit (C), and one of a variety of regulatory subunits that belong to four different families of unrelated proteins (e.g. B, B', B'', and B'''). These regulatory subunits control the localization and specific activity of different ABC heterotrimers within cells by directing the phosphatase activity to different substrates. Among various regulatory subunits, Zang *et al* showed that especially the B regulatory subunit is responsible for the p34^{SEI-1} stability (26). It is also well known that IR-induced multiple signaling pathways are controlled by phosphorylation and dephosphorylation of serine and threonine residues in proteins, which is mediated by different kinds of Ser/Thr kinases and phosphatases. For example, IR inactivates heterotrimeric PP2A in an ataxia telangiectasia-mutated (ATM)-dependent and wortmannin-sensitive manner causing the activation of cell cycle checkpoints (30,31).

These facts implied that PP2A might be responsible for the decreased p34^{SEI-1} expression in response to IR. Among many regulatory subunits, especially the B55 regulatory subunit unit was considered to be responsible for this mechanism. This assumption was based on the fact that IR has a negative effect especially on the B55 subunit but not on the B56 regulatory subunit. This hypothesis was at first tested by measuring the protein level of the B55 regulatory subunit of PP2A (PP2A-B55) and p34^{SEI-1} after the cells were exposed to IR. The level of the PP2A-B55 subunit was down-regulated to a great degree after the exposure to IR. It was followed by a decrease in the total amount of p34^{SEI-1} protein (Fig. 4A). Therefore, we concluded that inactivated PP2A due to the absence of the PP2A-B55 regulatory subunit no longer exerted a positive effect on the p34^{SEI-1} expression. This conclusion was supported by using OA, potent inhibitor of PP2A. The p34^{SEI-1} protein level was measured in the presence or absence of 0.1 mM of OA. The MCF-7 cells were treated with OA for 4, 8, 16 and 24 h. The level of p34^{SEI-1} protein did not change until 8 h but started to decrease from 16 h (data not shown). The p34^{SEI-1} protein level was significantly down-regulated at 24 h (Fig. 4B).

Taken together, our result suggests that IR-induced loss of PP2A-B55 seems to be responsible for the down-regulation of p34^{SEI-1} protein in MCF-7 cells.

Effect of hydrogen peroxide (H_2O_2) on p34^{SEI-1} expression in MCF-7 cells. Both IR and H_2O_2 belong to reactive oxygen species (ROS) producing DNA damage through reactive oxygen intermediates (32). Accordingly, we expected that H_2O_2 would have a similar effect on the p34^{SEI-1} expression in MCF-7 cells. To test this hypothesis, MCF cells were treated with different concentrations of H_2O_2 of 0.0, 0.2, 0.4, 0.8, 1.0,

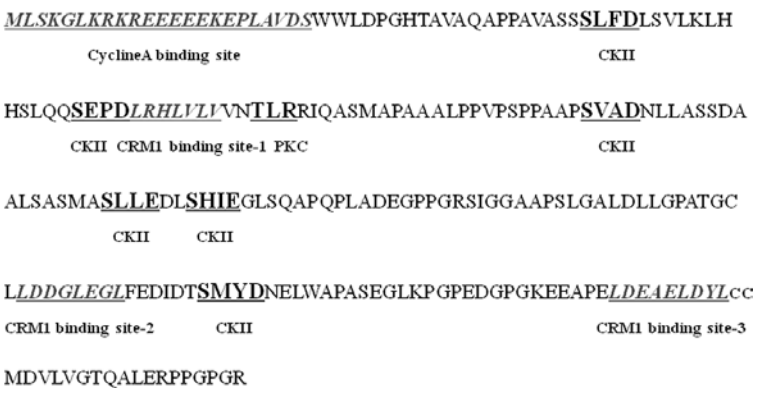

Figure 6. Putative motif search for Ser/Thr kinase binding sites in p34^{SEI-1} protein. Potential phosphorylation sites and responsible putative kinases were predicted using the TRANSFAC database (http://www.genome.ad.jp), which is shown in amino acid sequences of the p34^{SEI-1} protein. Phosphorylation sites containing serine and threonine resides, CKII and PKC binding sites, are shown as underlined and bolded. Putative cyclin A and CRM1 binding sites are underlined and italicized.

and 5.0 mM. Cell growth was repressed in cells treated with concentrations higher than 0.4 mM H_2O_2 but not in untreated control cells (Fig. 5A). The treatment of concentrations higher than 0.8 mM H_2O_2 caused cell death (Fig. 5B). Cells treated with 0.4 mM H_2O_2 were subjected to cell .cycle arrest at both G1 and G2, which was different from that of IR (Fig. 5C). For further study, 0.4 mM H_2O_2 was used to test whether H_2O_2 causes the same effect on the p34^{SEI-1} expression level. The total proteins and RNA were isolated from MCF-7 cells treated with 0.4 mM of H_2O_2 as indicated in Materials and methods. The expression level of the p34^{SEI-1} gene was measured at the protein and transcriptional levels using Western blotting and RT-PCR, respectively. Unexpectedly, no significant change in p34^{SEI-1} expression was detected at either level (Fig. 5D). This result implies that MCF-7 cells seem to use different signaling pathways in response to IR and H_2O_2 stresses although both of them belong to the same DNA damage-inducing stimuli of reactive oxygen species (ROS).

Discussion

In the process of identifying radiation-responsive proteins, our results showed that the level of p34^{SEI-1} expression was decreased in response to IR at the protein level but not at the transcriptional level. The p34^{SEI-1} expression is down-regulated at least partly due to the inhibition of PP2A phosphatase activity, which results from a decreased B55 regulatory subunit. Our result implies that the p34^{SEI-1} protein level is strongly affected by phosphorylation and dephosphorylation on protein. In an effort to find the dephosphorylatin site on the p34^{SEI-1} protein, we tried to identify the possible Ser/Thr protein kinase involved in the phosphorylaion of the p34^{SEI-1} protein by using the TRANSFAC database (http://www.genome.ad.jp). Our protein sequence-based analysis revealed seven putative binding sites of two kinds of Ser/Thr protein kinases, six serine residues in Casein kinase II (CKII) binding motifs and one threonine residue in protein kinase C (PKC) binding motifs in the p34^{SEI-1} protein (Fig. 6). CKII has been implicated in cell cycle control, DNA repair, regulation of the circadian rhythm, and other cellular processes. PKC controls the function of other proteins in many cellular processes. However, it was proposed that p34^{SEI-1} is phosphorylated only on serine but not on threonine residue (26). Therefore, PKC appears not to be a possible kinase for phosphorylation of the p34^{SEI-1} protein. In addition, Darwish *et al* suggested that the p34^{SEI-1} protein has a putative cyclin A binding site at N-terminal of the p34^{SEI-1} protein. This implies that the Cyclin A/CDK2 might be also responsible for the phosphoryation of p34^{SEI-1} (33). CDK2 (cyclin dependent kinase 2) also belongs to the Ser/Thr protein kinase superfamily. CDK2 activated by CyclinA plays a critical role in the control of the cell cycle at the G1/S (start) and G2/M (mitosis) transitions.

It has been suggested that p34^{SEI-2} (TRIP-Br2) can exist in a serine-phosphorylated form in cells. Phosphorylated p34^{SEI-2} is then translocated from the nucleus into the cytosol by the CRM1-mediated nuclear export machinery and targeted to be degraded by 26 proteasome (34). Considering that the p34^{SEI-1} belongs to the same family as p34^{SEI-2}, it is plausible to assume that the p34^{SEI-1} expression may be regulated in a similar manner. In fact, our protein analysis revealed three putative CRM1 binding sites in the amino acid sequences of p34^{SEI-1} protein (Fig. 6). CRM1 directs nuclear export of target proteins via direct binding to the leucine-rich nuclear-export signals (NES consensus) of amino acids (35,36). Based on all known facts and our study, we propose a model of the regulation of PP2A on p34^{SEI-1} expression in response to IR. Nuclear p34^{SEI-1} forms a complex with the E2F1/DP-1 transcriptional factors close to E2F-responsive promoters. The complex induces E2F-mediated transcription for proper G1/S transition. Phosphorylation of the E2F1/DP-1/p34^{SEI-1} transcriptional complexes by cyclin A/CDK2 or yet-unknown kinase(s) may lead to their release from E2F-responsive promoters. Dissociated p34^{SEI-1} may be targeted for ubiquitination by unknown ubiquitin ligase(s). Polyubiquitinated p34^{SEI-1} may be recognized by the CRM1-mediated nuclear export machinery as the case of p34^{SEI-2} and thus eventually exported to cytosol, where the polyubiquitinated p34^{SEI-1} maybe targeted to the 26S proteasome for proteolysis. In this process, if phosphorylated p34^{SEI-1} is dephosphorylated by PP2A, p34^{SEI-1} may not be degraded by proteasome. However, if cells are exposed to IR, PP2A will be inactivated because of the decreased PP2A-B55 regulatory subunit. Therefore, p34^{SEI-1} is no longer a dephosphorylated stable form and therefore expression of

p34^{SEI-1} is down-regulated in response to IR. Our study leads to the question whether IR induced G2 cell arrest has a direct relatioship with decreased levels of p34^{SEI-1} and PP2A-B55. Although substantial indirect and direct evidence points to the key roles of p34^{SEI-1} and PP2A in cell cycle regulation, the control of the enzyme activities prior to and after the transition are not fully clarified. Considering the important roles of p34^{SEI-1} in cell cycle regulation at the G2 phase as well as the G1/S phase, we speculate that nonfunctional p34^{SEI-1} and PP2A-B55 may not be able to normally regulate the cell cycle and therefore they may be at least in part related to G2 cell cycle arrest in response to IR.

In conclusion, our findings suggest that alleviating the positive effect of PP2A-B55 on p34^{SEI-1} oncogene could contribute to developing of more effective ways of combining radiation therapy with other systemic therapies. Moreover, this study will be helpful to better understand the molecular mechanism that responds to radiation in cancer cells.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MEST) and Sookmyung Women's University (2010).

References

1. El-Deiry WS: The role of p53 in chemosensitivity and radiosensitivity. Oncogene 22: 7486-7495, 2003.
2. Iliakis G, Wang Y, Guan J and Wang H: DNA damage checkpoint control in cells exposed to ionizing radiation. Oncogene 22: 5834-5847, 2003.
3. Eckardt-Schupp F and Klaus C: Radiation inducible DNA repair processes in eukaryotes. Biochimie 81: 161-171, 1999.
4. Iliakis G: Cell cycle regulation in irradiated and nonirradiated cells. Semin Oncol 24: 602-615, 1997.
5. Keyse SM: The induction of gene expression in mammalian cells by radiation. Semin Cancer Biol 4: 119-128, 1993.
6. El-Deiry WS: Regulation of p53 downstream genes. Semin Cancer Biol 8: 345-357, 1998.
7. Kasid U, Suy S, Dent P, Ray S, Whiteside TL and Sturgill TW: Activation of Raf by ionizing radiation. Nature 382: 813-816, 1996.
8. Robson T, Joiner MC, Wilson GD, McCullough W, Price ME, Logan I, Jones H, McKeown SR and Hirst DG: A novel human stress response-related gene with a potential role in induced radioresistance. Radiat Res 152: 451-461, 1999.
9. Waldman T, Lengauer C, Kinzler KW and Vogelstein B: Uncoupling of S phase and mitosis induced by anticancer agents in cells lacking p21. Nature 381: 713-716, 1996.
10. Amundson SA, Myers TG and Fornace AJ: Roles for p53 in growth arrest and apoptosis: putting on the brakes after genotoxic stress. Oncogene 17: 3287-3299, 1998.
11. Forrester HB, Vidair CA, Albright N, Ling CC and Dewey WC: Using computerized video time lapse for quantifying cell death of X-irradiated rat embryo cells transfected with c-myc or c-Ha-ras. Cancer Res 59: 931-939, 1999.
12. Lowe SW, Schmitt EM, Smith SW, Osborne BA and Jacks T: p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature 362: 847-849, 1993.
13. Maity A, McKenna WG and Muschel RJ: The molecular basis for cell cycle delays following ionizing radiation: a review. Radiother Oncol 31: 1-13, 1994.
14. Kis E, Szatmari T, Keszei M, Farkas R, Esik O, Lumniczky K, Falus A and Safrany G: Microarray analysis of radiation response genes in primary human fibroblasts. Int J Radiat Oncol Biol Phys 66: 1506-1514, 2006.
15. Kim W, Oe LS, Kim JS, Ryu YH, Byeon JY, Kim HJ, Kim YI, Heo JS, Park YM and Jung G: Comparison of proteome between hepatitis B virus- and hepatitis C virus-associated hepatocellular carcinoma. Clin Cancer Res 9: 5493-5500, 2003.
16. Janicke RU, Engels IH, Dunkern T, Kaina B, Schulze-Osthoff K and Porter AG: Ionizing radiation but not anticancer drugs causes cell cycle arrest and failure to activate the mitochondrial death pathway in MCF-7 breast carcinoma cells. Oncogene 20: 5043-5053, 2001.
17. Sugimoto M, Nakamura T, Ohtani N, Hampson L, Hampson IN, Shimamoto A, Furuichi Y, Okumura K, Niwa S, Taya Y and Hara E: Regulation of CDK4 activity by a novel CDK4-binding protein, p34(SEI-1). Genes Dev 13: 3027-3033, 1999.
18. Li J, Melvin WS, Tsai MD and Muscarella P: Nuclear protein p34SEI-1 regulates the kinase activity of cyclindependent kinase 4 in a concentration-dependent manner. Biochemistry 43: 4394-4399, 2004.
19. Bartek J, Bartkova J and Lukas J: The retinoblastoma protein pathway in cell cycle control and cancer. Exp Cell Res 237: 1-13, 1997.
20. Sherr CJ and Roberts JM: CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 13: 1501-1512, 1999.
21. Tang DJ, Hu L, Xie D, Wu QL, Fang Y, Zeng Y, Sham JS and Guan XY: Oncogenic transformation by SEI-1 is associated with chromosomal instability. Cancer Res 65: 6504-6508, 2005.
22. Ried T, Petersen I, Holtgreve-Grez H, Speicher MR, Schrock E, Du MS and Cremer T: Mapping of multiple DNA gains and losses in primary small cell lung carcinomas by comparative genomic hybridization. Cancer Res 54: 1801-1806, 1994.
23. Tang TC, Sham JS, Xie D, Fang Y, Huo KK, Wu QL and Guan XY: Identification of a candidate oncogene SEI-1 within a minimal amplified region at 19q13.1 in ovarian cancer cell lines. Cancer Res 62: 7157-7161, 2002.
24. Hong SW, Kim CJ, Park WS, Shin JS, Lee SD, Ko SG, Jung SI, Park IC, An SK, Lee WK, Lee WJ, Jin DH and Lee MS: p34SEI-1 inhibits apoptosis through the stabilization of the X-linked inhibitor of apoptosis protein: p34^{SEI-1} as a novel target for anti-breast cancer strategies. Cancer Res 69: 741-746, 2009.
25. Tian H, Faje AT, Lee SL and Jorgensen TJ: Radiation-induced phosphorylation of Chk1 at S345 is associated with p53-dependent cell cycle arrest pathways. Neoplasia 4: 171-180, 2002.
26. Zang ZJ, Gunaratnam L, Cheong JK, Lai LY, Hsiao LL, O'Leary E, Sun X, Salto-Tellez M, Bonventre JV and Hsu SI: Identification of PP2A as a novel interactor and regulator of TRIP-Br1. Cell Signal 21: 34-42, 2009.
27. Janssens V and Goris J: Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. Biochem J 353: 417-439, 2001.
28. Millward TA, Zolnierowicz S and Hemmings BA: Regulation of protein kinase cascades by protein phosphatase 2A. Trends Biochem Sci 24: 186-191, 1999.
29. Zolnierowicz S: Type 2A protein phosphatase, the complex regulator of numerous signaling pathways. Biochem Pharmacol 60: 1225-1235, 2000.
30. Guo CY, Brautigan DL and Larner JM: ATM-dependent dissociation of B55 regulatory subunit from nuclear PP2A in response to ionizing radiation. J Biol Chem 277: 4839-4844, 2002.
31. Guo CY, Brautigan DL and Larner JM: Ionizing radiation activates nuclear protein phosphatase-1 by ATM-dependent dephosphorylation. J Biol Chem 277: 41756-41761, 2002.
32. Zastawny TH, Kruszewski M and Olinski R: Ionizing radiation and hydrogen peroxide induced oxidative DNA base damage in two L5178Y cell lines. Free Radic Biol Med 24: 1250-1255, 1998.
33. Darwish H, Cho JM, Loignon M and Aoui-Jamali MA: Overexpression of SERTAD3, a putative oncogene located within the 19q13 amplicon, induces E2F activity and promotes tumor growth. Oncogene 26: 4319-4328, 2007.
34. Cheong JK, Gunaratnam L and Hsu SI: CRM1-mediated nuclear export is required for 26 S proteasome-dependent degradation of the TRIP-Br2 proto-oncoprotein. J Biol Chem 283: 11661-11676, 2008.
35. Kutay U and Guttinger S: Leucine-rich nuclear-export signals: born to be weak. Trends Cell Biol 15: 121-124, 2005.
36. Nigg EA: Nucleocytoplasmic transport: signals, mechanisms and regulation. Nature 386: 779-787, 1997.