Protein expression pattern in response to ionizing radiation in MCF-7 human breast cancer cells

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Received July 29, 2011; Accepted September 26, 2011

DOI: 10.3892/ol.2011.444

Abstract. Breast cancer is one of the most common types of cancer in women and is highly treatable by radiotherapy. However, repeated exposure to radiation results in tumor cell resistance. Understanding the molecular mechanisms involved in the response of tumors to γ-irradiation is important for improving radiotherapy. For this reason, we aimed to identify radiation-responsive genes at the protein level. In the present study, we observed differentially expressed proteins using 2D-PAGE and MALDI-TOF-MS for the global analysis of protein expression patterns in response to ionizing radiation (IR). When the expression patterns of proteins were compared to a control gel, numerous spots were found that differed greatly. Among them, 11 spots were found to be significantly different. One set of proteins (GH2, RGS17, BAK1, CCNH, TSG6, RAD51B, IGFBP1 and CASP14) was upregulated and another set of proteins (C1QRF, PLSCR2 and p34^{SE1-1}) was downregulated after exposure to γ-rays. These proteins are known to be related to cell cycle control, apoptosis, DNA repair, cell proliferation and other signaling pathways.

Introduction

Breast cancer is a common malignancy and a leading cause of death in women throughout the the world. Radiotherapy is considered crucial treatment for most common types of cancer and is usually used in conjunction with chemotherapy, hormone therapy or surgery. Radiation is known to activate multiple signaling pathways, causing cancer cells to become inactivated and resulting in diverse types of stress responses, including apoptosis, cell cycle arrest, senescence and gene induction. However, a large number of tumors fail to respond to radiotherapy as they become less sensitive or more resistant to radiation after consecutive treatments. Various studies on the molecular mechanisms of resistance to radiotherapy have been carried out. However, obstacles related to overcoming this resistance remain to be solved. Therefore, identification of the radiation-responsive genes may aid to better understand the molecular mechanisms involved in the response of tumors to radiation and, ultimately, improve radiotherapy.

A number of aspects of the initial response to radiation-induced DNA damage have been extensively analyzed via p53 and other DNA damage checkpoint responses (1,2). For example, the tumor suppressor gene *TP53* (*p53*) plays a significant role in the cellular response to radiation-induced stress (3-5). In cells carrying a non-functional *p53*, cell-cycle arrest and DNA repair cannot occur. This increases the level of genomic instability and allows tumor growth despite the exposure to radiation (4-6). In addition, a number of radiation-responsive genes are identified through different approaches. Various stress-responsive effector genes have been known to be inducible by radiation (7-11). The major effector genes identified in the radiation-induced response include *RAF1, CDKNIA (p21), GADD45A (GADD45)*, 14-3-3 σ (a member of the *YWHA* family), *BAX, TNFRSF (Fas/APO1)*, *TNFRSF 10B (KILLER/DR5)*, *PIG*, *THBS1 (TSP1)*, *IGFBP3* and *DIR1*. These radiation-responsive effector genes and their control factors play key roles in the cellular response to radiation-induced stress by modulating cell cycle checkpoints, apoptosis and DNA repair, which enhance cell survival (3,8-10,12-16). Changes in the mRNA expression levels were reported by cDNA microarray data targeting radiation-responsive genes in human breast cancer cells (17-21). In addition, Kis *et al* identified radiation-responsive genes using a microarray analysis in primary human fibroblasts (22). These authors detected approximately 200 ionizing radiation (IR) responsive genes at the transcriptional level, of which 30 (28 up- and 2 downregulated) responded to radiation in all investigated cells and 20 were grouped according to function: DNA damage response (GADD45A, BTG2, PCNA and IER5), regulation of the cell cycle and cell proliferation (CDKN1A, PPM1D,

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Abbreviations: IR, ionizing radiation

Key words: breast cancer, ionizing radiation, 2D-PAGE

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) (22). However, the mRNA expression profiles did not match their protein expression profiles as the radiation-responsive genes appeared to respond differently at the transcriptional and protein levels. Moreover, the molecular mechanisms inactivating tumor cells in response to radiation have yet to be elucidated, although several mechanisms are known to be involved in this process. We, therefore, aimed to identify the radiation-responsive genes at the protein level using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and matrix assisted laser desorption/ionization time of flight-mass spectrometry (MALDI-TOF-MS) in MCF-7 human breast cancer cells. Tools such as 2D-PAGE and MALDI-TOF-MS have been widely used for the study of cancer proteomics, as noted in several other studies (23-25).

In this study, a global analysis of the protein expression pattern was performed using 2D-PAGE and MALDI-TOF-MS to identify radiation-responsive proteins in MCF-7 breast cancer cells.

Materials and methods

Condition of the MCF-7 cell culture and treatment of ionizing radiation (IR). The MCF-7 human breast cancer cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, 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) (WelGENE Inc., Daegu-si, Korea). This medium was supplemented with 10% fetal bovine serum (Gibco BRL, Seoul, 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). The cells were harvested after the indicated time of incubation at 37˚C. Cell viability was assessed by a trypan blue exclusion test.

Protein extraction. Cells were washed with cold phosphate-buffered saline (PBS) and centrifuged at 3,000 rpm, 4˚C for 3 min. The centrifuged 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 (5X) on ice, and then centrifuged at 13,000 rpm, at 4˚C for 10 min. The concentrations of the protein samples were determined using a modified Bradford protein assay (Bio-Rad, Hercules, CA, USA).

2D-PAGE, gel scanning and image analysis. The first dimensional isoelectric focusing (IEF) was performed on precast 18 cm immobilized pH 3.0-10.0 gradient (IPG) strips (Amersham Pharmacia Biotech) at 20˚C using a commercial flatbed electrophoresis system (IPGphor; Amersham Pharmacia Biotech). 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 as a tracking dye. The mixtures were loaded onto an IPG strip, followed by 12 h of active rehydration at 50 V and 50 μA, which was ramped to 500 V and 50 μA over a period of 10 min. It was then maitained at 5000 V and 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, SDS-PAGE was performed for separation without a stacking gel at 150 V, and 20 mA for 20 h per gel in a SDS-electrophoresis buffer (25 mM Tris, 92 mM glycine and 0.1% SDS). After electrophoresis separation, the gels were stained using Bio-Rad Silver Stain kit (catalog no. 161-0443). The stained 2-D gels were scanned on a Las-3000 (Fuji Photo Film Co.) using 2-D software PDQuest (Bio-Rad). The different gel patterns were then automatically matched to each other and the quantities of the matched spots in the different gels were compared. The molecular masses and pI values were calculated with 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 the gels were identified by MALDI-TOF-MS. After tryptic in-gel digestion of 2D-PAGE resolved proteins, samples for MALDI peptide mass mapping were prepared, as previously described (26). Mass spectra were obtained using a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems, Cambridge, MA, USA). The proteins were identified according to their tryptic peptide mass fingerprint after a database search was performed on MS-Fit, which is accessible over the World Wide Web at http://prospector.ucsf.edu/. MS-Fit performed a rapid database search by comparing experimentally determined masses from the proteolytic digestion of proteins with peptide database masses calculated from the NCBlnr protein database and Swiss-Prot accession numbers.

Cell cycle analysis by fluorescence-activated cell sorting (FACS). For a FACS analysis, cells were harvested at the indicated time points, washed twice in ice-cold PBS, 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. The cell pellets were resuspended in 0.5 ml of 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 their 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 the CellQuest 3.2 software (Becton-Dickinson).

Results

G2 cell cycle arrest induced by IR in MCF-7 cells. MCF-7 cells were irradiated with different doses of γ-radiation of 1, 5, 10, or 20 Gy, in which the cell growth was repressed in the cells exposed to 20 Gy of radiation, as shown in Fig. 1A. The 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 the FACS analysis (Fig.1B). This result suggests that IR-irradiated MCF-7 cells undergo cell cycle arrest rather than apoptosis. These data are consis-

Table I. Standard proteins identified by MALDI-TOF-MS.

AN[a]	Gene	Protein	pI/Mw (Da)
P02545	*LMNA*	Lamin A/C	6.6/74140
Q08209	*PPP3CA*	Serine/threonine protein phosphatase 2B catalytic subunit, α isoform	5.6/58688
P31942	*HNRPH3*	Heterogeneous nuclear ribonucleoprotein H3	6.4/36927
Q8N1Q1	*CA13*	Carbonic anhydrase XIII	6.5/29443
Q13886	*BTEB1*	Transcription factor BTEB1	8.8/27235
Q9NY72	*SCN3B*	Sodium channel β-3 subunit	4.7/24703
20977872[b]	*ZFX*	X-linked zinc finger protein	3.9/18903
P05092	*PPIA*	Peptidyl-prolyl cis-trans isomerase A	7.7/18013
Q9H503	*C20ORF179*	Hypothetical BAF-like protein C20orf179	5.5/10309

[a]AN, Swiss-Prot accession number (AN) from the Swiss-Prot database. [b]Accession number is from the NCBInr protein database.

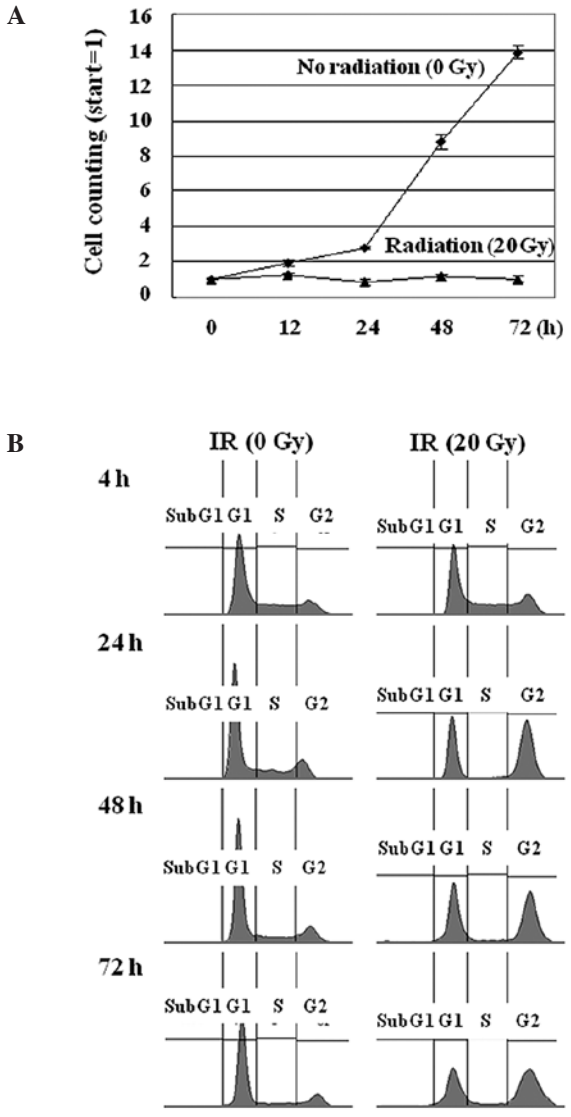


Figure 1. Ionizing radiation (IR)-induced cell cycle arrest at the G2 phase in MCF-7 cells. (A) Cell growth analysis of MCF-7 cells in response to IR. MCF-7 cells were exposed to 20 Gy of IR, incubated for the indicated times, and the growth rate was then checked. (B) FACS analysis of MCF-7 cells either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

tent with previous studies in which IR induced cell cycle arrest but failed to activate the mitochondrial death pathway in MCF-7 cells (27). Thus, to establish the genes responsible for this phenotype we aimed to identify the radiation-responsive proteins in MCF-7 cells.

Identification of radiation-responsive genes in MCF-7 cells. To obtain information on the expression profile of radiation-induced and reduced protein, MCF-7 breast cancer cells were irradiated with 20 Gy of γ-rays and harvested after incubation for 0 , 4 , 12 , 24 or 48 h. We then observed differentially expressed proteins using 2D-PAGE and MALDI-TOF-MS as described in Materials and methods. Proteins with approximately 1,000-1,200 spots in each gel were visualized by silver staining and detected by PDQuest 2D-image-analysis software (Bio-Rad). After the spot detection, the gels were matched to each other, with the aid of the so-called landmark function, and approximately 730 spots in total were matched in all gels analyzed according to the 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 included LMNA, PPP3CA, HNRPH3, CA13, BTEB1, SCN3B, ZFX, PPIA and C20ORF179 (Fig. 2 and Table I), and were used for the generation of a relevant pI and Mr scale for the entire pattern.

When the expression patterns of proteins were compared to the control gel, numerous spots were found to differ greatly from one another. Among them, 11 spots were found to be significantly different. One set of proteins was upregulated in response to IR in the MCF-7 cells. These proteins included RAD51B, CCNH, TSG6, GH2, RGS17, BAK1, IGFBP1 and CASP14 (Table II). Fig. 3 shows the regions containing upregulated proteins, which are marked by black rectangles and are magnified on the lower gels. All of these proteins showed a similar pattern in terms of the protein expression level. The expression level of each protein started to increase after exposure to γ-rays (Fig. 3A-C). 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.

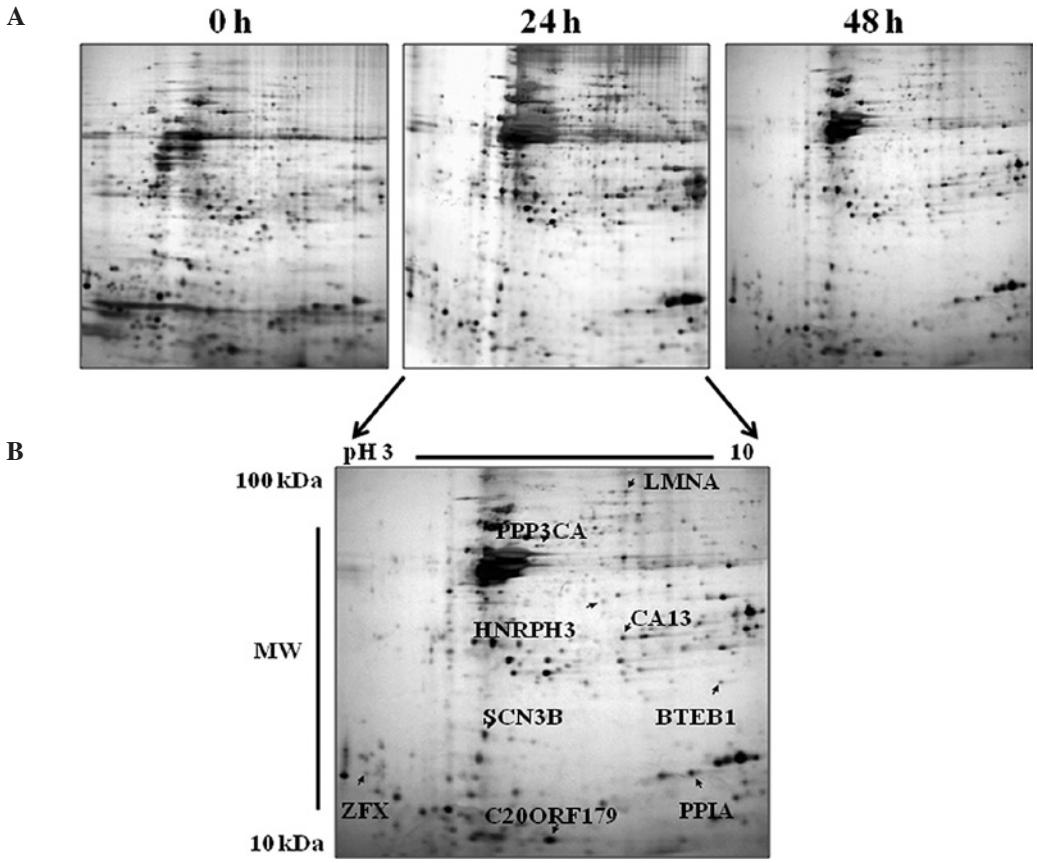
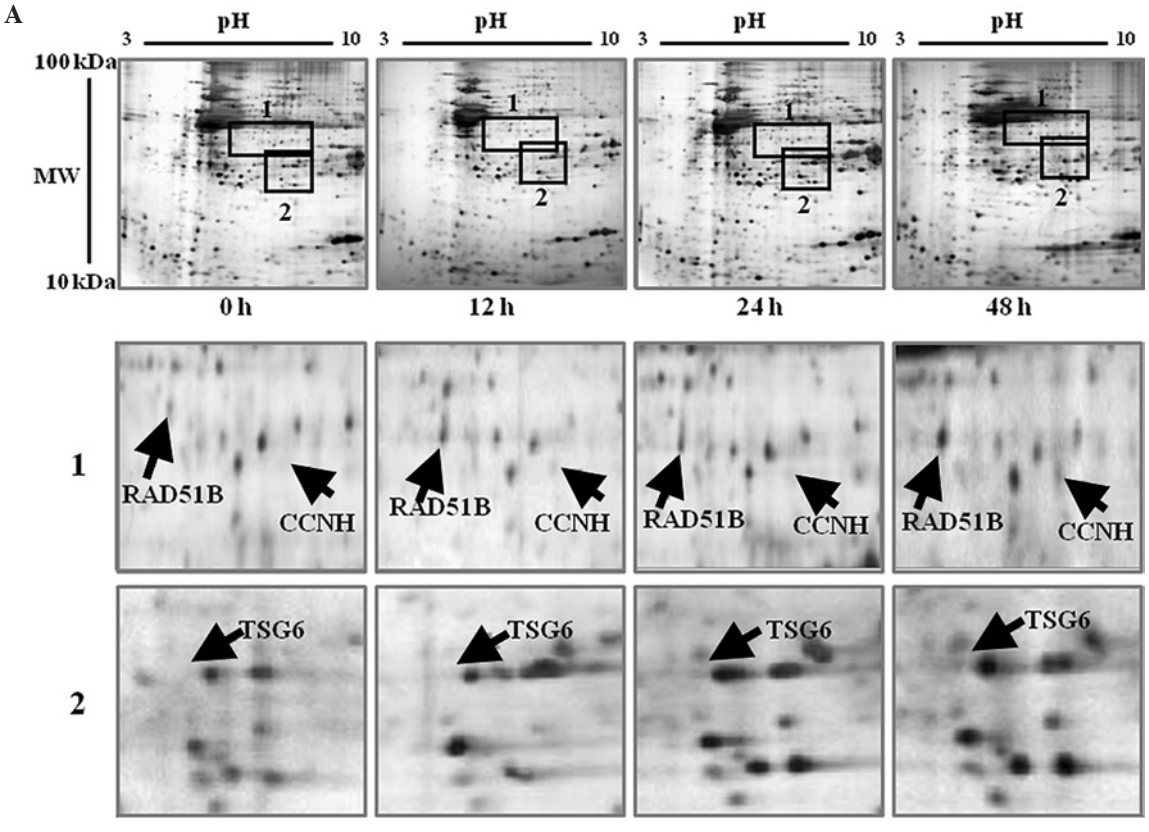


Figure 2. Position and identification of the selected standard proteins in the silver-stained 2-D gels. (A) MCF-7 cells were treated with 20 Gy of IR and incubated for the indicated times. The proteins are shown as a silver-stained image. (B) The standard protein spots are shown in an enlarged gel. More specific information regarding the spots is provided in Table I. The horizontal axis is the isoelectric focusing dimension, which stretches from pH 3.0 (left) to pH 10.0 (right). The vertical axis is the polyacrylamide gel dimension, which stretches from 10 kDa (bottom) to 100 kDa (top).



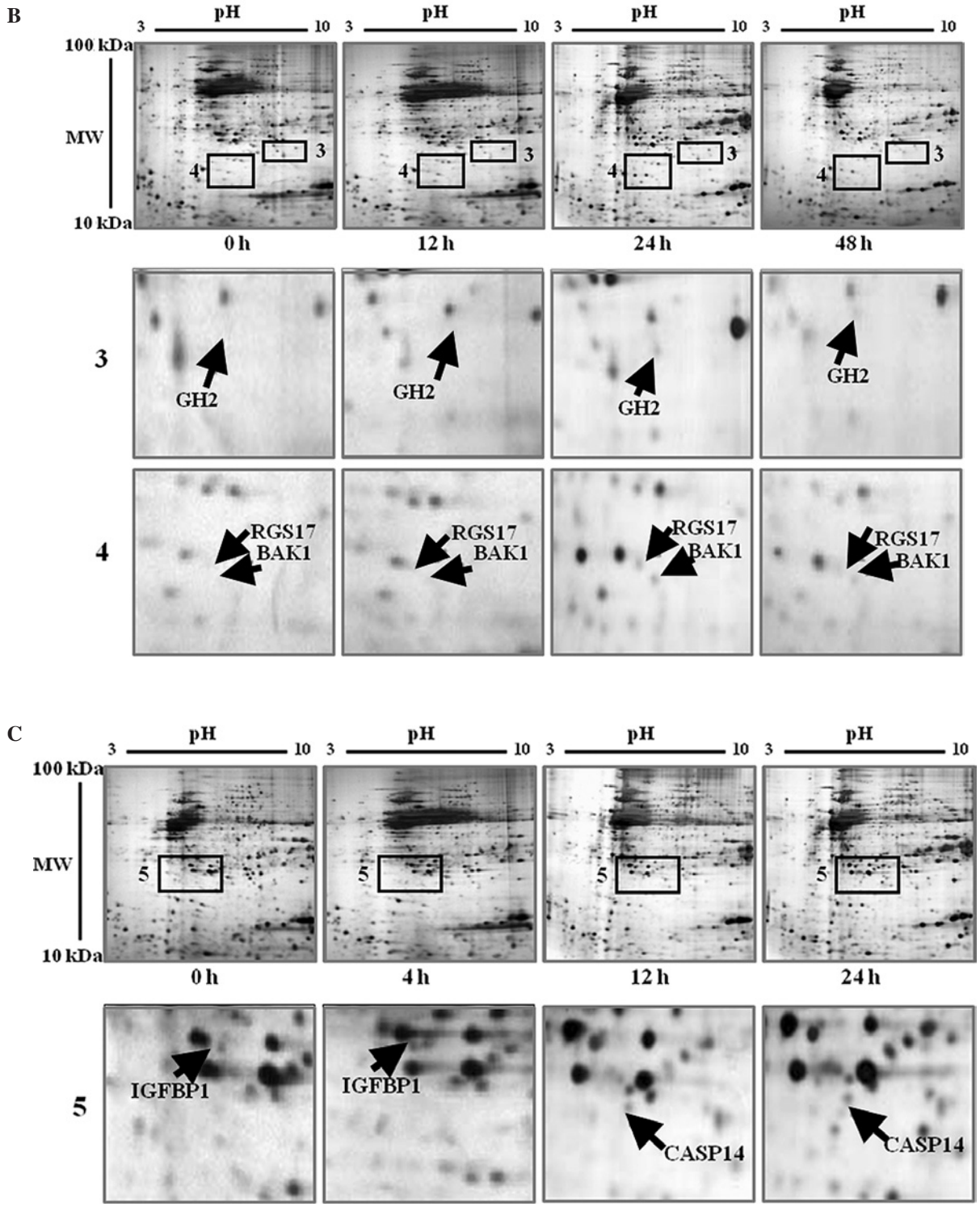


Figure 3. Up-regulated proteins following exposure to ionizing radiation (IR). MCF-7 cells were treated with 20 Gy of IR and incubated for the indicated times. The proteins are shown as a silver-stained image. The horizontal axis is the isoelectric focusing dimension, which stretches from pH 3.0 (left) to pH 10.0 (right). The vertical axis is the polyacrylamide gel dimension, which stretches from 10 kDa (bottom) to 100 kDa (top). In the uppermost panels, protein expression patterns are shown, in which the black rectangles of the upper gels include upregulated protein spots. In the lower enlarged gels, upregulated proteins are indicated with the arrows on the enlarged gels, which show the differences more clearly. Differentially expressed protein spots identified by peptide mass fingerprinting are labeled here with the protein name. More specific information about the spots (A) RAD51B, CCNH and TSG6; (B) GH2, RGS17 and BAK1; and (C) IGFBP1 and CASP14 is provided in Table II.

In another set, the expression levels of three proteins were downregulated following exposure to γ-rays. They include C1QRF, PLSCR2 and p34^{SE1-1}. These proteins are marked by black rectangles on the gels and are magnified on the lower gels (Fig. 4). In the percentage volume of the spots compared between 0 and 48 h using the PDQuest software, a considerable amount of each protein was detected in the control gel (0 h). The C1QRF and PLSCR2

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

Class	AN[a]	Gene	Protein	pI/Mw (Da)[b]
Class I	(Upregulated proteins in response to IR in MCF-7 cells)			
	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*	Regulator 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 (Downregulated proteins in response to IR in MCF-7 cells)				
	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 (AN) from the Swiss-Prot database. [b]pI and Molecular weights were calculated using PDQuest software by utilizing the selected pI and Mr standard proteins.

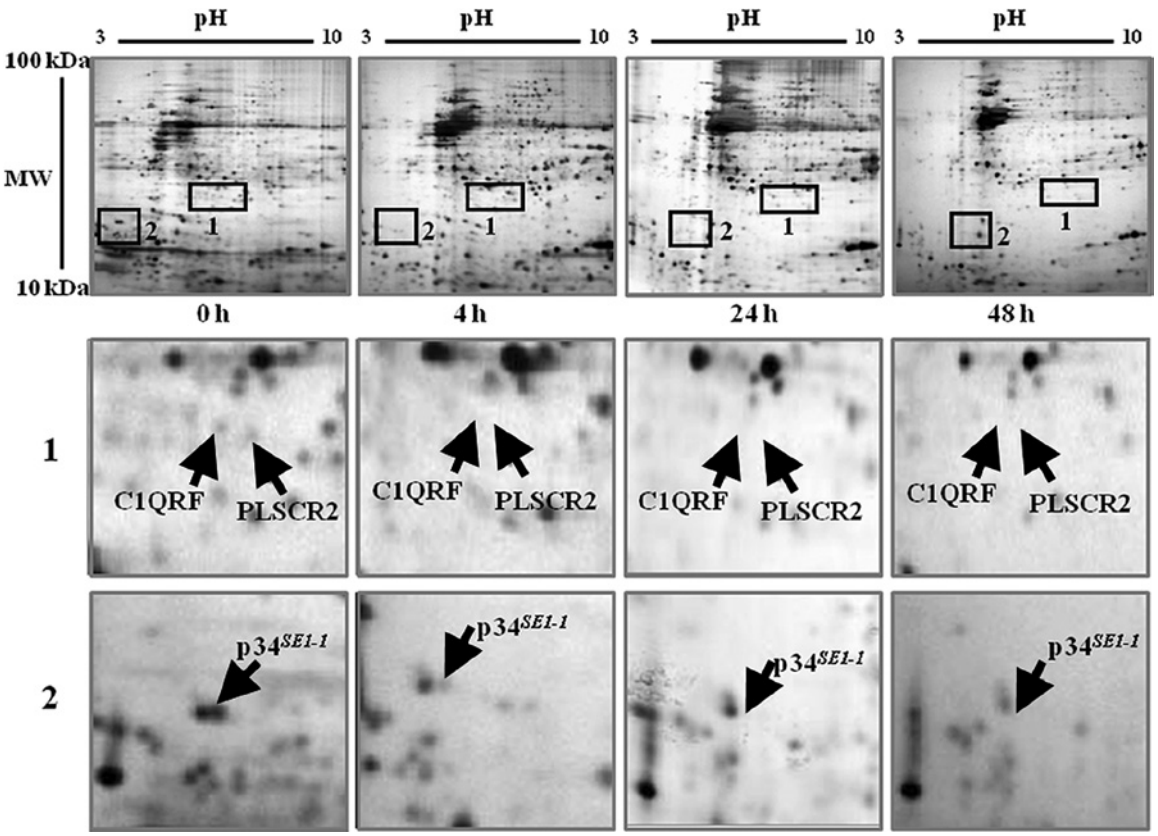


Figure 4. Downregulated proteins following exposure to ionizing radiation (IR). MCF-7 cells were treated with 20 Gy of IR and incubated for the indicated times. The proteins are shown as a silver-stained image. In the upper panels, the regions containing downregulated proteins are indicated with the black rectangles. In the lower enlarged gels, downregulated proteins are indicated with the arrows. More specific information about the spots C1QRF, PLSCR2 and p34^{SE1-1} is provided in Table II.

proteins were found only at 0 h and p34^{SE1-1} was detected at 0 and 4 h. However, their expression levels started to decrease from 12 h and were significantly downregulated after 48 h (Fig. 4).

Discussion

In this study, we observed up- and downregulated proteins in the MCF-7 breast cancer cell line. The proteins maintained

at an elevated level in the radiation-derived MCF-7 cells are correlated to cell cycle control, apoptosis, DNA repair, and cell proliferation. The genes responsible for this elevation include: *RAD51B*, which encodes a DNA repair protein RAD51 homolog 2, which is involved in the homologous recombination repair (HRR) pathway of double-stranded DNA breaks arising during DNA replication or induced by DNA-damaging agents (28). *CCNH* encodes cyclin H, which regulates CDK7, the catalytic subunit of the CDK-activating kinase (CAK) enzymatic complex. It is involved in cell cycle control and RNA transcription by RNA polymerase II. Its expression and activity are constant throughout the cell cycle (29). *TSG6* encodes the tumor necrosis factor-inducible protein TSG-6. This gene may be involved in cell-cell and cell-matrix interactions during inflammation and tumorigenesis (30). *GH2* encodes a growth hormone variant that plays an important role in growth control and stimulates the proliferation of human MCF-7 mammary carcinoma cells by activating the MAPK signaling pathway (31). *RGS17* encodes a regulator of G-protein signaling 17 that inhibits signal transduction by increasing the GTPase activity of G protein α subunits thereby driving them into their inactive GDP-bound form. *RGS17* plays important roles in T-cell proliferation and IL-2 production and RGS17 deficiency leads to impaired T-cell activation (32). *BAK1* encodes a Bcl-2 homologous antagonist/killer that has been reported to be regulated by p53 (33). It induces cell death and accelerated apoptosis (34-36). *IGFBP1* encodes insulin-like growth factor binding protein 1. IGFBP1 is expressed in the breast both *in vitro* and *in vivo* (37,38). The upregulation of IGFBP1 is associated with the malignant transformation of breast tissue (38). CASP14 encodes caspase-14 which is involved in the death receptor and granzyme B apoptotic pathways. It may function as a downstream signal transducer of cell death (39).

On the other hand, protein expression levels for C1QRF, PLSCR2 and $p34^{SE1-1}$ were decreased. The genes responsible in this case include: *C1QRF*, which encodes a C1q-related factor containing one C1q domain that is a component of the complement pathway, and is involved in tumor cytotoxicity (40). Moreover, *PLSCR2* encodes phospholipid scramblase 2, which plays a central role in the recognition of apoptotic and injured cells by the reticuloendothelial system (41). $p34^{SE1-1}$ encodes a transcriptional regulator interacting with PHD-bromodomain 1 (Trip-Br1), which renders the activity of cyclin D1/CDK4 resistant to the inhibitory effects of $p16^{INK4a}$. In addition, $p16^{INK4a}$ specifically binds and inhibits CDK4, the partner kinase of the D1 cyclin strongly implicated in the phosphorylation of pRb and thereby in G1/S control (42-44). The decreased expression of the $p34^{SE1-1}$ protein may be related to the induction of cell cycle arrest at the G2 phase in MCF-7 cells.

In conclusion, our study may aid in better understanding the molecular mechanism that responds to radiation in cancer cells. Additionally, our findings may contribute to the development of more effective ways of combining radiation therapy with other systemic therapies.

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