

Two-Dimensional Electrophoretic Analysis of Radio Frequency Radiation-Exposed MCF7 Breast Cancer Cells

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Radio frequency (RF) radiation/2-dimensional electrophoresis/MCF7 cells.

Although many *in vitro* studies have previously been conducted to elucidate the biological effects of radio frequency (RF) radiation over the past decades, the existence and nature of any effects is still inconclusive. In an effort to further elucidate this question, we have monitored changes in protein expression profiles in RF-exposed MCF7 human breast cancer cells using two-dimensional gel electrophoresis. MCF7 cells were exposed to 849 MHz RF radiation for 1 h per day for three consecutive days at specific absorption rates (SARs) of either 2 W/Kg or 10 W/kg. During exposure, the temperature in the exposure chamber was kept in an isothermal condition. Twenty-four hours after the final RF exposure, the protein lysates from MCF cells were prepared and two-dimensional electrophoretic analyses were conducted. The protein expression profiles of the MCF cells were not significantly altered as the result of RF exposure. None of the protein spots on the two-dimensional electrophoretic gels showed reproducible changes in three independent experiments. To determine effect of RF radiation on protein expression profiles more clearly, three spots showing altered expression without reproducibility were identified using electrospray ionization tandem mass spectrometry analysis and their expressions were examined with RT-PCR and Western blot assays. There was no alteration in their mRNA and protein levels. As we were unable to observe any significant and reproducible changes in the protein expression profiles of the RF radiation-exposed MCF7 cells using high throughput and non-high throughput techniques, it seems unlikely that RF exposure modulates the protein expression profile.

INTRODUCTION

As a result of the exponential increase in mobile phone use over recent years, public concerns regarding the potential harmful effects of the radio frequency (RF) radiation emitted from these devices are also growing. A variety of studies have already been performed concerning the possible biological and health effects of mobile phone radiation. Various *in vitro* studies designed to determine the effects on DNA damage, chromosome aberration, cell cycle distribu-

tion, cell proliferation, cell survival, stress response, and gene expression as endpoints of observation have been conducted.^{1–8)} However, there still remains a great deal of uncertainty regarding the biological effects of RF radiation. It has been suggested that high throughput screening proteomics techniques could be utilized to identify a variety of potential molecular targets of RF radiation.⁹⁾ The potential molecular targets obtained via proteomic analysis might be applied in further experiments targeted toward ascertaining the possible biological effects of RF radiation.

It has been reported that the mobile phone radiation has non-thermal effect on the heat shock protein 27 (HSP27) and p38 mitogen activated protein kinase (MAPK) in EA.hy926 human endothelial cells, according to the results of 2-dimensional electrophoresis (2-DE).¹⁰⁾ The same group provided lists of genes and proteins which evidenced statistically significantly altered expression in the EA.hy926 and the EA.hy926v1 variant,⁹⁾ and identified 8 proteins that were statistically significantly affected in forearm's skin in human volunteers who were exposed to RF radiation.¹¹⁾ Two other groups published papers showing positive or negative effect of RF radiation on protein expression profiles in human lens

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epithelial cells or MCF7 cells, respectively.^{12,13)} Despite the power of proteomic analysis to screen up to a thousand of proteins at once, only a few groups have thus far reported results of proteomics to elucidate RF radiation effects on protein expression profiles. Thus, we decided to apply proteomic analysis techniques to RF radiation exposed cells in order to gain insight into whether RF radiation might exert any biological effects *in vitro*.

In this study, we exposed MCF7 human breast cancer cells to 849 MHz RF radiation at average SAR values of either 2 W/kg or 10 W/kg, and monitored the changes in protein expression profiles via 2-DE high throughput technique, and validated some protein spots which showed altered expression without reproducibility in triplicate using non-high throughput techniques.

MATERIALS AND METHODS

Cell culture

Human breast cancer cell line, MCF7 was purchased from the American Type Culture Collection (ATCC) (Manassas, VA USA). Cells were plated in 100 mm culture dishes at densities of 5×10^4 cells and grown in DMEM medium supplemented with 10% heat-inactivated FBS (WelGENE Inc., Daegu, Korea), 100 $\mu\text{g}/\text{ml}$ streptomycin, and 100 U/ml penicillin in a 5% CO_2 incubator at 37°C. For the heat shock treatment, culture plates containing cells were tightly wrapped with parafilm and immersed in a water bath at 43°C

for 30 minutes.

Radiofrequency exposure system

The exposure system designed for this study is a rectangular-cavity-type. The details about exposure system are described previously.¹⁴⁾ Block diagram and cavity structure of the exposure system are shown in Fig. 1. A real CDMA signal at 849 MHz (channel 779) is applied to the chamber after amplification.

RF radiation and ionizing radiation exposure

After 24 h of cell seeding at densities of 5×10^4 cells in 100 mm culture dish, cells were exposed to 849 MHz RF radiation in 100 mm Petri dishes. Petri dishes were placed within the exposure chamber, at the position shown in Fig. 1B. The exposure system was thermally equilibrated for 30 minutes before exposure to RF. RF radiation exposure was conducted at SAR values of 2 or 10 W/kg for 1 hr per day for three consecutive days. During the exposure, the temperature in the chamber was maintained at $37.03 \pm 0.08^\circ\text{C}$ (M \pm SD) for sham-exposure, at $37.14 \pm 0.07^\circ\text{C}$ for 2 W/kg RF radiation-exposure, and at $37.17 \pm 0.08^\circ\text{C}$ for 10 W/kg RF radiation-exposure, by circulating water within the cavity. Temperature of the culture media was monitored twice a second throughout the exposure period in sham exposed, 2 W/kg, or 10 W/kg exposed groups. Typical temperature histories during RF exposure at 2 W/kg and 10 W/kg were described in our previous work.¹⁴⁾ After each exposure, the

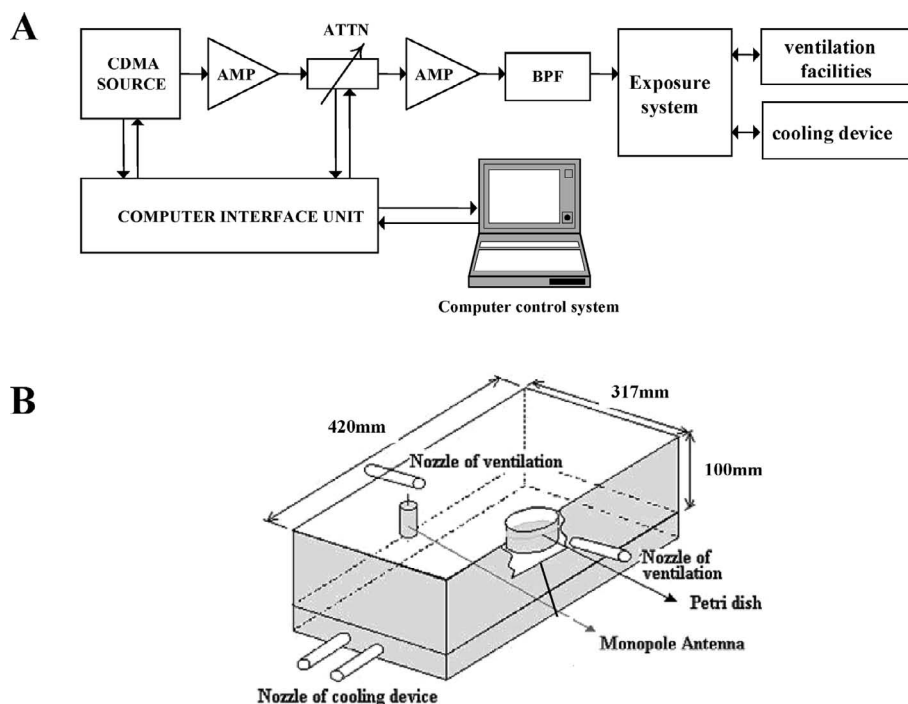


Fig. 1. Rectangular cavity type of cell exposure system. A: Block diagram of the exposure system. B: Cavity structure of exposure chamber.

cells were immediately transferred to a cell culture incubator. The culture media were changed 24 h after the first exposure, and the cells were harvested 24 h after the final exposure (about 80% cell confluence). For the sham exposure, the cells were kept in the RF radiation device, but were not exposed to RF radiation.

For the positive control group experiments, cells were exposed to 0.5 Gy of gamma radiation from a ^{137}Cs gamma ray source (Atomic Energy of Canada Ltd., Mississauga, Ontario, Canada) at a dose rate of 3.81 Gy/min and kept in CO_2 incubator for additional 24 h (about 80% cell confluence) for the preparation of cell lysates.

Preparation of protein lysates for two dimensional electrophoresis

Cells were washed with ice-cold PBS buffer twice and harvested by centrifugation at 1,200 rpm for 5 min. Lysis buffer (9 M Urea, 2 M Thiourea, 100 mM DTT, 2% CHAPS (w/v), 60 mM *n*-octyl-D-glucopyranoside, 2% IPG buffer (pH 3–10, Amersham biosciences, Piscataway, NJ) containing protease inhibitor), was added and the cells gently suspended. Cells were incubated on the ice for 30 min and centrifuged at 14,000 rpm, 10 min, 4°C. The supernatant was taken for the protein quantification with Bradford assay Reagent (Bio-Rad, Hercules, CA) as described in manufacturer's protocol, and subjected to the subsequent 2-dimensional gel electrophoresis.

Two-dimensional electrophoresis

Overall procedure has followed as previously described¹⁵⁾ with slight modification. Isoelectric focusing of each sample containing equal amount of protein was conducted on a linear wide-range immobilized pH gradient (pH 3–10; 24 cm-long immobilized pH gradient [IPG] strips) with a total focusing time of 81,780Vhr, at 20°C, using the IPGphor system in accordance with the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ). The second dimension was then conducted on lab-made SDS-PAGE gels (11% polyacrylamide, 0.26% 1,4-Bis (acryloyl) piperazine (PDA)/ 25.5 cm × 19.6 cm × 1 mm), under constant current, in three steps (Step 1: 5 w/gel; Step2: 10 w/gel; step 3: 15 w/gel) at 20°C, using an Ettandalt 6 system (Amersham Biosciences). The analytical gels were stained using the PlusOne™ silver staining kit (Amersham Biosciences) in accordance with the manufacturer instructions, with the exception of the glutaraldehyde treatment. Spot intensities were quantified by the 2-D image program Progenesis version 2005 (Nonlinear USA Inc, Durham, NC, USA).

In-gel protein digestion and electrospray ionization tandem mass spectrometry (ESI-MS/MS)

Proteins were subjected to in-gel trypsin digestion. The excised gel spots were then destained and incubated using 200 mM ammonium bicarbonate for 20 min. These gel

pieces were dehydrated and dried, and then rehydrated. The peptide solution was desalted with a C_{18} nano column (IN2GEN Co., Ltd., Seoul, Korea). The MS/MS of peptides generated via in-gel digestion was conducted by nano-ESI on a Q-TOF2 mass spectrometer (Micromass, Manchester, UK). The product ions were analyzed with an orthogonal TOF analyzer. The data were processed with a Mass Lynx Windows NT PC system. In order to identify the proteins, all MS/MS spectra recorded on tryptic peptides derived from the spots were searched against protein sequences from the NCBI databases, using the MASCOT search program (www.matrixscience.com).

Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was prepared using TRIzol reagent (Invitrogen, Calsbad, CA) and used as a template for cDNA synthesis with M-MLV RT using SuperScript™ III reverse transcriptase kits (Invitrogen, Calsbad, CA). Subsequently, PCR were performed by 20 to 27 cycles. The forward and reverse primer sequence were as follows: GRP78 forward 5'-AAG CCC GTC CAG AAA GTG TT-3', reverse 5'-TTG GTC ATG ACA CCT CCC AC-3'; Glucosidase II α forward 5'-GGA ACC TGG GGC TGT ATG TT-3', reverse 5'-GCA ACC TGG GGC TGT ATG TT-3'; Pin1 forward 5'-TAA TGG CAC TGG TGG CAA GT-3', reverse 5'-CTT GCC ATC CAA CCA CTC AG-3', β -actin forward 5'-CAA GAG ATG GCC ACG GCT-3', reverse 5'-TCC TTC TGC ATC CTG TCG GCA-3'. Primers were obtained from Bioneer Corporation (Daejeon, Korea). The final RT-PCR products were electrophoresed on 1.5% agarose gel, stained with 0.5 g/ml ethidium bromide solution, and visualized on a UV transilluminator (Bio-Rad Laboratories, Hercules, CA).

Western blotting

Cells were lysed in extraction buffer (25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 0.25% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride), incubated on ice for 30 min. The lysate was centrifuged and quantitated with a Bradford Assay Reagents (Bio-Rad, Hercules, CA). Equal amount of proteins was loaded onto 10% SDS-PAGE gel. After separation of proteins depending on their molecular weights, it was transferred to a nitrocellulose membrane. The membrane blot was incubated with specific primary antibodies at 4°C overnight, and washed three times in PBST. Protein bands were detected by sequential treatment with an HRP-conjugated secondary antibodies and an enhanced chemiluminescence substrate kit (Amersham, Arlington Heights, IL). Antibodies of GRP78 (SC-13968), Glucosidase II α (C-16), Pin1 (G-8), β -actin (SC-1616) were obtained from Santa Cruz Biotech, Inc. (Santa Cruz, CA, USA).

RESULTS AND DISCUSSION

In order to elucidate whether exposure to RF radiation elicits alteration in protein expression profiles, we conducted two-dimensional electrophoretic (2-DE) analyses. MCF7 human breast cancer cells were exposed to 849 MHz RF radiation at average SAR values of 2 W/kg or 10 W/kg for 1 hr per day for three consecutive days. To exclude the thermal effects of RF radiation, cooling water was circulated through the cavity of exposure chamber and the temperature of the culture media was maintained within the range of $37 \pm 0.3^\circ\text{C}$ during the exposure period. After the completion of the exposure period, the cells were maintained in a CO_2 incubator for 24 hours at 37°C prior to the preparation of the protein lysates. The protein lysates were prepared from sham-exposed, 2 W/kg RF radiation-exposed, and 10 W/kg RF radiation-exposed MCF7 cells, and subjected to 2-DE. Figure 2 shows a representative 2-DE gel image of control MCF7 cells. The X-axis and Y-axis indicate the pI and molecular weight ranges, respectively. In order to compare protein spots in detail among the groups (i.e., sham-, 2 W/kg RF radiation-, and 10 W/kg RF radiation-exposed MCF7 cells), the 2-DE images were divided into quarters. The left upper (A), left lower (B), right upper (C), and right lower (D) quadrants of the representative gels are shown in Fig. 3. In order to estimate the reproducibility, 2-DE was conducted in triplicate (i.e., each sample obtained from independent sample preparations ran once on a 2-DE, three times). We

detected no significant and reproducible alterations in protein expression patterns between the sham-exposed and RF radiation-exposed cells.

Though no spots showed reproducible changes in triplicate, we found three spots which showed altered expression once among three independent experiments either in 2 W/kg-exposed or in 10 W/kg-exposed cells (Fig. 4). Therefore, we wanted to confirm clearly whether RF radiation exposure could affect on levels of these proteins using non-high throughput techniques. Firstly, the spots were identified as glucose-regulated protein, 78 kDa (GRP78), peptidylprolyl isomerase A (PIN1), and glucosidase II by electrospray ionization tandem mass spectrometry (ESI MS/MS) analysis. Next, their mRNA and protein levels were confirmed with semi-quantitative RT-PCR and Western blot analyses. We observed no difference in mRNA levels of GRP78, PIN1, and glucosidase II (Fig. 5A). When amplification of RNA reaches a plateau, RNAs initially present in different levels may be detected as equal intensities of PCR products. Therefore, we examined a number of cycles ranging from 20 to 27. Their protein levels were not changed in RF-exposed cells in independent two samplings (Fig. 5B). To show the sensitivity of the 2-DE technique, we performed positive control experiments. Ionizing radiation exposure (0.5 Gy) and heat treatment (43°C , 30 min) which were employed as a positive controls caused reproducible alterations in the protein expression profiles of the MCF-7 cells 24 h after each treatment in two independent experiments (Supplementary Fig. S1).

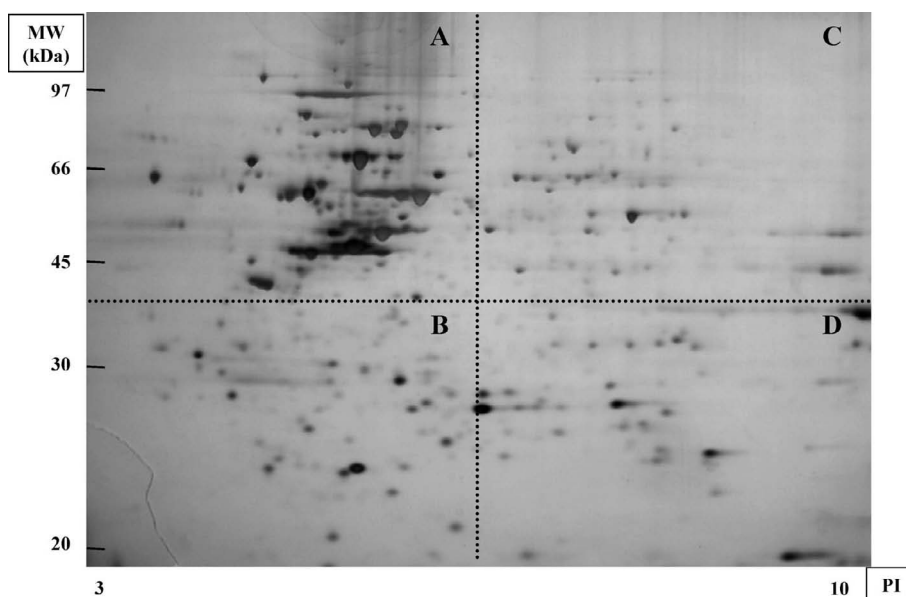


Fig. 2. Two-dimensional electrophoresis profiles of control MCF 7 human breast cancer cells. Protein lysate was focused on an 24 cm 3–10 IPG strip, separated by SDS-polyacrylamide electrophoretic gel (1 mm thick 11% polyacrylamide gel), and then stained by silver nitrate. X axis and Y axis indicates pI range molecular weight range, respectively.

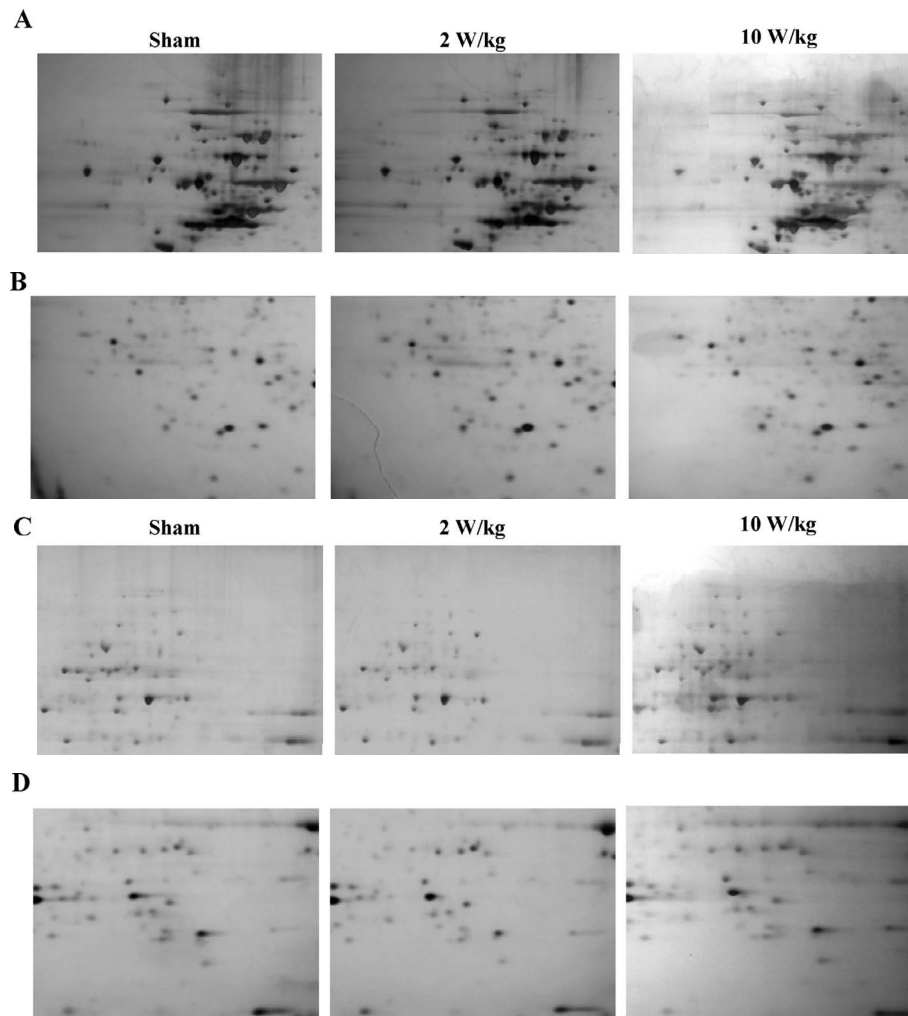


Fig. 3. Two-dimensional electrophoresis profiles of RF radiation-exposed MCF7 cells. MCF7 cells were exposed to 849 MHz RF radiation at either 2 W/kg or 10 W/kg average SAR value for 1 hr with 23 hrs interval three times, successively. After 24 hrs, two-dimensional electrophoresis were conducted, and stained with silver nitrate. To compare protein spots in detail among the groups, sham-, 2 W/kg RF radiation-, and 10 W/kg RF radiation-exposed MCF7 cells, gel images were divided into one fourth as shown in Fig. 3A and show the areas of left upper (A), left lower (B), right upper (C), right lower (D).

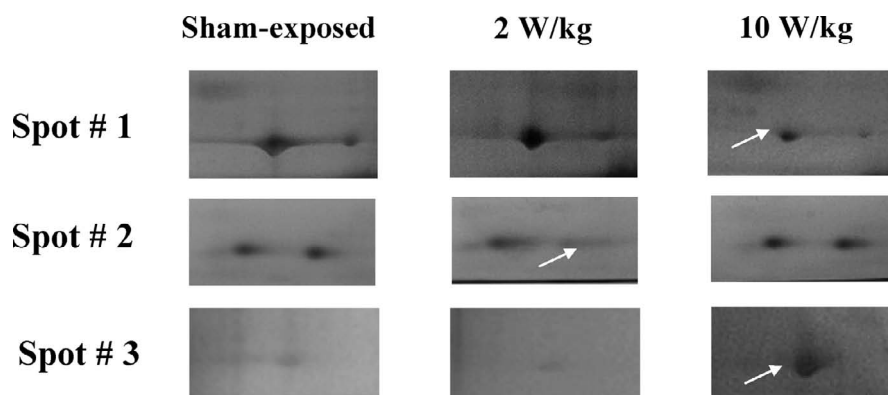


Fig. 4. Protein spots showing differential expressions after exposure of RF radiation without reproducibility in three independent experiments.

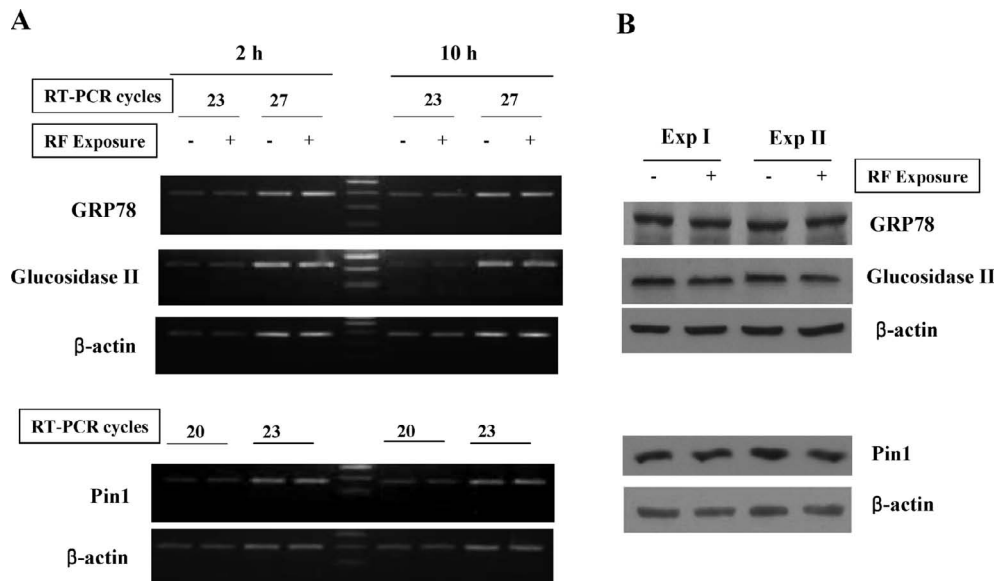
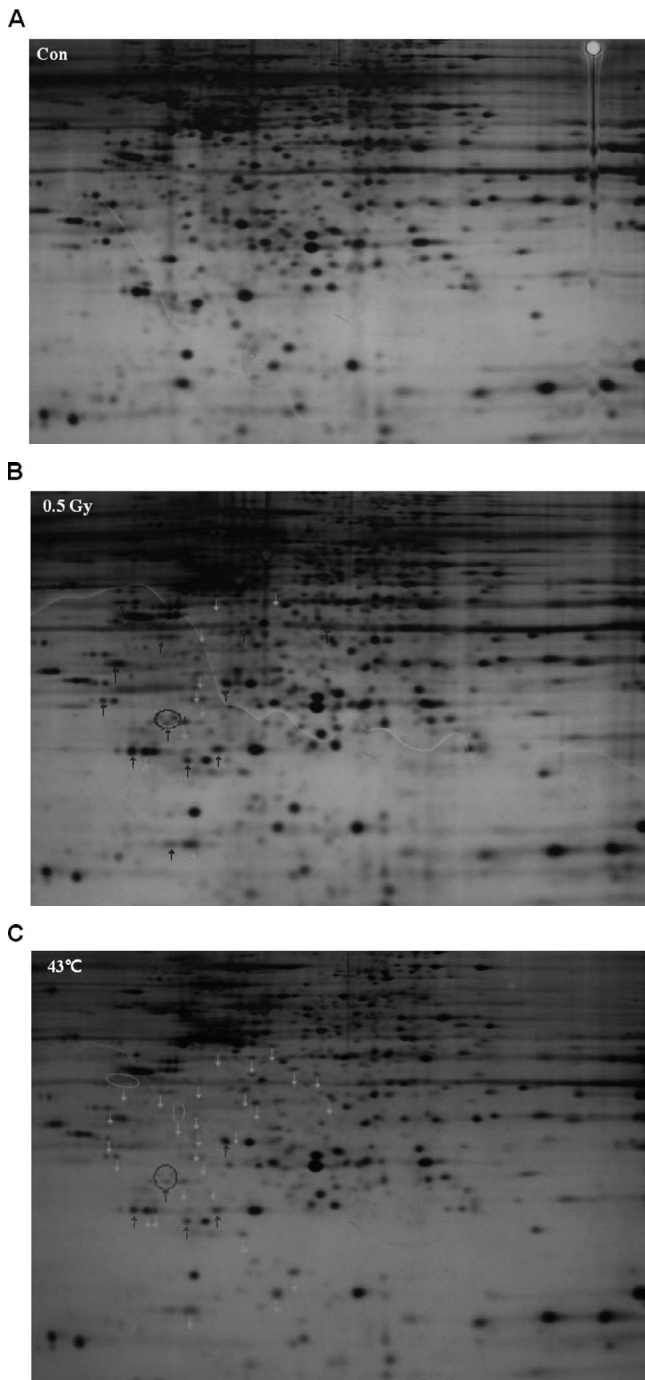


Fig. 5. Validation of RF radiation effect on GRP78, glucosidase II, and Pin1 with non-high-throughput methods, semi-quantitative RT-PCR (A) and Western blot analysis (B). GRP78 and glucosidase II were exposed to 10 W/kg, and Pin1 was exposed to 2 W/kg. Cycles ranging from 20 to 27 were adopted to avoid reaching a plateau of amplification reaction of RT-PCR. Exp I and Exp II designate two independent experiments.

Whereas ionizing radiation, ultraviolet, and DNA-damaging agents have already been determined to exert devastating effects on the human body, causing irreversible cellular damage, RF radiation may not be sufficiently strong enough to cause any harmful effects on normal cellular functions, as has been reported in some previous studies.¹⁶⁾ However, despite numerous attempts to determine whether RF radiation emitted by mobile phones could induce biological effects, the uncertainty still exists. Recently developed high-throughput screening methods, including genomic, transcriptomic, and proteomic techniques, are powerful tools for the detection of alterations in more than thousands of genes or proteins simultaneously. These methods allow for the identification of putative target molecules affected in specific signaling pathways. High-throughput screening methods are suitable for the evaluation of the biological effects of RF radiation.¹⁷⁾ Among the high-throughput screening methods available, proteomics is the most direct analytical method, because it demonstrates what is actually occurring within the cell and not what might be happening as predicted with microarrays. Also, it identifies important post-translational modifications elucidating additional protein function. Despite recent technical developments in differential quantitative proteomics via stable isotope-labeling with amino acids in cell culture and isotope-coded affinity tag techniques, 2-DE remains the method of choice for differential proteomics, due to several advantages. Firstly, the protein spots generated by this technique can be readily visualized via silver nitrate staining, and annotated via MALDI and TOF-MS or ESI-MS/MS.^{15,18)} The putatively identified pro-

teins will be further studied, in order to understand the mechanisms underlying a variety of biological effects. The 2-DE technique also makes it possible to distinguish between two proteins that differ in only a single charged amino acid. Therefore, the proteomic technique can be considered an appropriate tool for the elucidation of the molecular effects of RF radiation. However, although proteomics provides sufficient resolution for the protein-level analysis of radiation-associated proteins, only a scant few proteomic analyses thus far have been conducted in the context of RF radiation research.

To the best of our knowledge, three research groups have published papers involving proteomics and RF radiation exposure. It has been reported that mobile phone exposure was shown to non-thermally cause a transient increase in the phosphorylation of HSP27 and the protein expression levels of HSP27 and p38 MAPK in EA.hy926 cells exposed to 900 MHz mobile phone radiation in Finland.¹⁰⁾ Authors hypothesized that mobile phone radiation may facilitate the development of brain cancer and may induce an increase in blood-brain barrier permeability, via the activity of HSP27. Same group observed that three proteins, including vimentin, were differentially expressed as the result of RF radiation exposure, using 2-DE in the same cell line.⁹⁾ As increases in the expression of F-actin, HSP27, and vimentin were observed, the authors inferred that the cytoskeleton might be one of the cytoplasmic structures that responds to mobile phone radiation. Finland research group further examined the response to mobile phone radiation using the EA.hy926 and EA.hy926v1 cell line.¹⁹⁾ Thirty eight spots evidenced



Supplementary Fig. S1. Two-dimensional electrophoresis profiles of control (A), 0.5 Gy of ionizing radiation (IR)-exposed (B), and 43°C heat-treated (C) MCF7 cells. After 24 hrs of IR or heat shock treatments, two-dimensional electrophoresis were conducted, and stained with silver nitrate. Arrows designate reproducibly up-regulated and down-regulated protein spots due to the exposure to either IR or heat in two independent experiments of each treatment.

statistically significant alterations in the EA.hy926 cells, and 45 spots in the EA.hy926v1 cells. Therefore, the group concluded that different types of cells and different species might respond differently to mobile phone radiation, and that this could explain, at least in part, the discrepancies in the studies conducted at different laboratories. Recently, they reported that 8 proteins were statistically significantly affected by the RF radiation exposure (1.3 W/kg) in forearm's skin of 10 female volunteers.¹¹⁾ Second group published that heat-shock protein (HSP) 70 and heterogeneous nuclear ribonucleoprotein K (hnRNP K) were detected as upregulated in human lens epithelial cells which were exposed to 1800 MHz GSM like radiation.¹²⁾ However, Zeng group reported that systematic proteomics of MCF7 cells revealed that a few but different proteins were differentially expressed under RF radiation exposure, implying that the observed effects might have occurred by chance.¹³⁾

In this study, we applied proteomics techniques to RF radiation-exposed MCF7 cells in order to identify differentially expressed RF-responsive proteins and their biological function. No apparent changes with reproducibility were detected in the protein expression profiles in triplicate. On the basis of our experience in the proteomics field, reproducibility among the independent experiments is the most important factor in determining and clarifying changes in protein expression profiles.^{15,20,21)} Therefore, we conducted three independent sampling and 2-DE to identify spots that were reproducibly altered due to the RF exposure. None of those spots evidenced reproducible alterations over all three independent experiments. Finally, we wanted to check the possibility that three spots showing altered expression without reproducibility in three independent experiments could be affected by the exposure of RF radiation. Those spots identified as GRP78, Pin1, glucosidase II showed no difference in mRNA and protein levels as shown in Fig. 5. From our results, it is concluded that no reproducibly upregulated or downregulated spots resulted from exposure to RF radiation and the reproducibility is important factor to get the certainty from the proteomics analysis.

Despite the many advantages of proteomic techniques, the methods require refinement in order to overcome certain technical limitations. The 2-DE technique can resolve a thousand spots on a two-dimensional protein map. However, as the number of intracellular proteins may be in excess of 30,000, the 2-DE technique is actually capable of detecting only abundantly expressed protein spots. The second limitation is the production of false-positive results due to problems in reproducibility and variability between experiments.¹⁷⁾ It has been discussed that there are certain limitations for the application of proteomics technologies despite of recent advancements and automation.²²⁾ General limitations are in sample preparation, protein abundance, 2D gel reproducibility, protein quantification, protein hydrophobicity and heterogeneity, protein size and charge and etc.

Therefore, as a further precaution against variability between experiments, putative target molecules must be validated using non-high-throughput screening methods, as previously suggested by Leszczynski *et al.*²³⁾ The relevant issues with regard to the applicability of proteomics techniques in RF radiation studies have been well summarized in the report published by Leszczynski and Meltz.²⁴⁾ Whitehead *et al.*²⁵⁾ reported that the number of genes changing expression after RF exposure does not exceed the false-positive rate. False positive rate in proteomics has been reported yet.

Proteomic analyses have also been previously utilized to elucidate the effects of extremely low frequency (ELF) radiation exposure on protein expression profiles. HL-60 cells exposed to 60 Hz ELF radiation evidenced a significant increase in HSP90 and HSP27 expression, as shown by 2-DE.²⁶⁾ Li *et al.*²⁷⁾ reported on the positive effects of ELF radiation exposure on protein expression profiles. However, Nakasono *et al.*²⁸⁾ concluded that no high-confidence changes occurred in either proteins or genes in *Saccharomyces cerevisiae* exposed to 50 Hz magnetic fields. In the field of proteomic analysis research, the effects of RF radiation and ELF radiation on protein expression profiles remain a matter of some controversy. More studies will be required in order to definitively settle the question as to whether non-ionizing radiation can induce certain biological effects.

In conclusion, we conducted comparative proteomic analyses to evaluate differences in expression occurring between sham-exposed and RF radiation-exposed MCF7 cells. RF radiation results in no reproducible alteration in protein expression under our exposure and analytical conditions. However, there is a possibility that minor changes in protein expression profile might be undetectable under current experimental techniques.

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