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# Proteomic Analysis of the Response of Human Endothelial Cell Line EA.hy926 to 1800 GSM Mobile Phone Radiation

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## Abstract

**Background:** We have earlier shown that exposure of human endothelial cell line EA.hy926 to 900 MHz GSM mobile phone radiation causes changes in the expression of numerous proteins. Here, we have examined the effects of 1800 MHz GSM mobile phone signal on the proteome of the same cell line.

**Results:** EA.hy926 cells were exposed for one hour to 1800 MHz GSM signal, simulating mobile phone talking conditions, at an average specific absorption rate (SAR) of 2.0 W/kg at 37±0.3°C. Sham samples were produced simultaneously in the same conditions but without the radiation exposure. Cells were harvested immediately after 1-hour exposure to the radiation, and proteins were extracted and separated using 2-dimensional electrophoresis (2DE). In total, 10 experimental replicates were generated from both exposed and sham samples. About 900 protein spots were detected in the 2DE-gels using PDQuest software and eight of them were found to be differentially expressed in exposed cells ($p<0.05$, t-test). Three out of these eight proteins were identified using Maldi-ToF mass spectrometry (MS). These proteins are: spermidine synthase (SRM), 78 kDa glucose-regulated protein (55 kDa fragment) (GRP78) and proteasome subunit alpha type 1 (PSA1). Due to the lack of the availability of commercial antibodies we were able to further examine expression of only GRP78. Using SDS-PAGE and western blot method we were not able to confirm the result obtained for GRP78 using 2DE. Additionally, we have not seen any effect of 1800GSM exposure on the expression of vimentin and Hsp27 - proteins that were affected by the 900 MHz GSM exposure in our earlier studies.

**Conclusions:** Our results suggest that the 900GSM and 1800GSM exposures might affect the expression of some proteins in the EA.hy926 cell line. The observed here discrepancy between the expression changes of GRP78 detected with 1DE and 2DE confirms the importance of validation of the results obtained with 2DE using other methods, e.g. western blot.

**Abbreviations:** 2DE: Two-dimensional electrophoresis; CHAPS: 3-[(3- Cholamidopropyl)dimethylammonio]-1-propanesulfonate; Da: Dalton; $ddH_2O$: Double distilled water; DMEM: Dulbecco's Modified Eagle's Medium; DTT: Dithioreitol; EA.hy926: Human endothelial cell line; ECL Enhanced chemiluminescence; GSM: Global System for Mobile Communications; HAT: (mixture of) sodium hypoxanthine, aminopterin, and thymidine; HRP: Horseradish peroxidase; IAA: Iodoacetamide; IEF: Isoelectric focusing; IPG: Immobilized pH gradient; LR: Linear-reflectron; MALDI-TOF: Matrix-assisted laser desorption/ionization time of flight; MS: Mass spectrometry/ mass spectrometer; $NH_4HCO_3$: Ammoniumbicarbonate; PAGE: Polyacrylamide gel electrophoresis; PBS: Phosphate buffered saline; pI: Isoelectric point; PMF: Peptide mass fingerprint; PMSF: Phenylmethylsulphonyl fluoride; PVDF: Polyvinylidene Fluoride; RF-EMF: Radiofrequency modulated electromagnetic field; SAR: Specific absorption rate; SDS: Sodium dodecyl sulphate; Tris-HCl: Tris(hydroxymethyl)aminomethane hydrochloride; Versene: Chelating agent containing EDTA

## Background

The use of mobile phones has widely increased over the past decade. However, the issue of potential health effects induced by mobile phone radiation remains controversial and further research is needed to fill-up the existing gaps in the knowledge about the biological and physiological effects of this low-level energy radiation.

We have proposed that the use of high-throughput screening techniques of transcriptomics and proteomics, as tools to find genes and proteins responding to mobile phone radiation, might help the process of finding out whether mobile phone radiation might cause any health risk (Leszczynski and Joenväärä, 2001; Leszczynski, 2006; Leszczynski and Meltz, 2006). Proteomics approach has been so far used only in a few *in vitro* studies (Leszczynski et al., 2002; Leszczynski et al., 2004; Nylund and Leszczynski, 2004; Nylund and Leszczynski, 2006; Zeng et al., 2006; Li et al., 2007) and in a single *in vivo* human volunteer study (Karinen et al., 2008). Such a small number of published studies does not allow for making any generalized conclusions about of the possible effects of mobile phone exposures on the cell proteome and on the cell physiology. Only by performing more of this kind of studies, the proteomic database can be

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expanded and, with the help of that, the impact of mobile phone radiation on cell proteome will be possible to assess.

We have previously determined that the 900 MHz GSM mobile phone radiation signal alters expression of several tens of proteins in the human endothelial cell line EA.hy926 (Leszczynski, et al., 2002; Nylund and Leszczynski, 2004; Nylund and Leszczynski, 2006). In the present study we have examined whether the 1800 MHz GSM mobile phone radiation signal exposure will also affect protein expression in EA.hy926 cells. Protein expression was determined using 2DE proteomics and results were compared with the earlier study that used 900 MHz GSM mobile phone radiation.

## Materials and Methods

### In Vitro Cell Model and Cell Culture Conditions

Brain capillary endothelial cells are one of the potential targets of the mobile phone radiation. In some animal studies it has been shown that mobile phone radiation might affect function of the blood-brain barrier. That is why we have selected to examine *in vitro* effects of mobile phone radiation on endothelial cells. Human endothelial cell line EA.hy926 was selected because of the uniformity of cell cultures from batch to batch and because of easy and fast means to generate large quantities of cells for experiments. Neither of the above is possible to achieve with primary endothelial cells, known for slow growth and for the variability between batches isolated from different human donors.

Human endothelial cell line EA.hy926 (a gift from Dr. Cora-Jean S. Edgell North Carolina University at Chapel Hill, NC, USA) was grown in Dulbecco's MEM (DMEM), supplemented with antibiotics, 10% foetal bovine serum, L-glutamine and HAT-supplement (Sigma, USA). For the mobile phone radiation experiments, cells were removed from culture flasks by brief trypsinization, washed in cell culture medium and seeded at a density of $0.4x10^6$cells/dish in 35 mm-diameter Petri dishes (NUNC, Denmark). After an overnight culturing the semi-confluent monolayers of EA.hy926 were exposed to mobile phone radiation or sham exposed.

### Exposure to Mobile Phone Radiation Signal

The sXc-1800 exposure system, developed and provided by the IT'IS Foundation and installed at STUK (Helsinki), was employed (Figure 1). This consists of two identical exposure chambers mounted in the same cell culture incubator. It is fully automated and enables exposures of cells in monolayers (H-polarization or at H-field maximum of the standing wave) at freely programmable amplitude modulations. The exposure chambers are based on resonant R18 waveguides, allowing for SAR values of several hundred W/kg at the cell monolayer level with a few watts input power. The identical environmental conditions (temperature, humidity, $CO_2$) are achieved in both exposure chambers because the inlet of the airflow to both chambers is at the same location. The system monitors, every 10 seconds, the incident field strengths, the proper functioning of the ventilators, the outlet air temperatures and the functional state of the whole exposure set-up. The Pt100 temperature sensors (accuracy ±0.1 °C) have been calibrated prior to the installation and the recorded differences in temperature are well

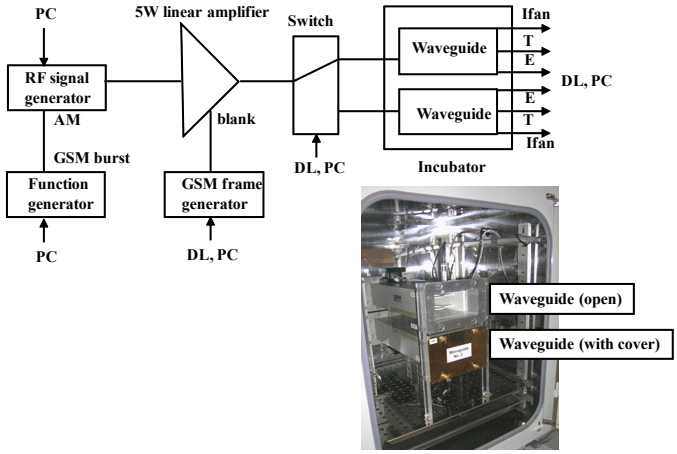


**Figure 1:** A diagram of sXc1800 mobile phone radiation exposure system (E: E-field sensors, T: temperarure sensors, Ifan: fan current sensors, DL: data logger i/o, PC: personal computer via GPIB) and photo of the waveguides inside a cell culture incubator.

within the specified long-term stability of the calibration. The induced temperature load due to mobile phone radiation absorption has been characterized as a function of SAR (t) for different signals and volumes of medium. This enables a reliable estimate of the maximum temperature rise as a function of the exposure. The ambient electromagnetic field of the cell culture incubator was measured in several positions within the incubator using an EFA-3 field measurement system (Wandel & Goltermann, Germany). Further details of the exposure system are described elsewhere (Schuderer et al., 2004). The signal applied in this study was GSM Talk. GSM Talk signal is characterized by a random change between the discontinuous transmission mode (DTX) and non-DTX or GSM Basic phases. The distribution in time was exponential with a mean duration of 10.8 seconds for non-DTX and 5.6 seconds for DTX. The dominant modulation components of this signal are 2, 8, 217, 1733 Hz, and higher harmonics. The more detailed description of the signal can be found elsewhere (Tillmann et al., 2006).

After overnight cultivation, the semi-confluent monolayers of EA.hy926 cells were placed in two 6-dish holders and inserted into the exposure chambers. In one of the exposure chambers, randomly selected by the system's computer, the cells were exposed to an average SAR of 2.0 W/kg at 37±0.3°C (to assure examination of non-thermal effects), while in the other chamber they were sham-exposed, in the similar conditions but without mobile phone radiation signal exposure. Precise control of the temperature of the cell cultures during the exposure to mobile phone radiation is of paramount importance to assure that the temperature increases are not responsible for the observed effects. Therefore, because in our experiments the temperature of cell cultures did not increase by more than 0.3°C we can state that the observed effects are of non-thermal nature (are not caused by any significant temperature increase). The experiments were performed in the blinded manner and the code was broken after the files from the exposure system were sent to IT'IS, Zurich, Switzerland.

### Protein Extraction

Immediately after the end of the 1-hour exposure cells were

quickly washed with PBS and harvested with versene. Proteins were extracted with a buffer consisting of 8 M Urea, 1 M Thiourea, 4% Chaps, 10 mM DTT, 2% IPG buffer pH 4-7, 1 mM sodium orthovanadate and 1 mM PMSF. Protein concentrations were measured using Bradford method. The 250µg of total protein was used for two-dimensional gel electrophoresis (2DE).

**2DE**

The isoelectric focusing was performed using an IPGphor apparatus (GE Healthcare, USA) and 24 cm long ready IEF strips pH 4-7 (GE Healthcare). The samples were loaded using in-gel rehydration in a buffer containing 9 M Urea, 2% Chaps, 0.2% DTT, 0.5% IPG buffer pH 4-7 for 4 hours. IEF was run at 20°C using step-and-hold methods as follows: 50 V 8 h; 100 V 1 h; 500 V 1 h; 1000 V 1 h; 2000 V 1 h; 8000 V until 95000 Vhrs were achieved. Before SDS-PAGE the IEF strips were equilibrated for 15 min with 6 M urea, 30% glycerol, 50 mM Tris-HCl, 2% SDS, and 10 mg/mL DTT and then for another 15 min in the same buffer, in which DTT was replaced by 25 mg/mL iodoacetamide (IAA). SDS-PAGE was run in 10% gel using Ettan DALTsix Electrophoresis system (GE Healthcare) at the constant power setting of 3.5W/gel for the first 0.5 hours and then 13W/gel. After electrophoresis the gels were silver stained. Gels were fixed (30% ethanol, 0.5% acetic acid), washed with 20% ethanol and ddH2O, sensitized with sodium thiosulfate (0.2 g/L), incubated in the silver nitrate solution (2 g/L) and developed (potassium anhydride 30 g/L, 37% formaldehyde 0.7 mL/L, sodium thiosulfate 0.01 g/L). The development was stopped with Tris 50 g/L + 0.5% acetic acid, and then the gels were washed twice with ddH2O and scanned.

**Data Analysis**

The silver stained gels were scanned using GS-710 densitometer (Bio-Rad, USA) and analyzed using PDQuest 7.2 software (Bio-Rad). In total, ten gels from both sham and exposed samples were analysed. The normalized spot volumes of the proteins from sham and exposed sample gels were statistically analyzed using student *t*-test at the confidence level of 95%. Protein spots, that visually appeared as technical artefacts (e.g. background areas of silver staining, irregular-shaped dust particles, air bubbles) but were erroneously detected by the software, were manually removed from the analysis.

**In-gel Digestions for Mass Spectrometry Protein Identification**

Proteins of interest were extracted from several gels and in-gel digested. Before digestion the proteins were reduced with 20 mM DTT in 0.1M ammonium-bi-carbonate ($NH_4HCO_3$) and alkylated with 55 mM IAA in $NH_4HCO_3$. Proteins were digested overnight at +37°C with modified trypsin (sequencing grade modified trypsin, porcine, Promega, USA) in 50 mM $NH_4HCO_3$. After overnight digestion, resulting peptides were extracted from gels with 25 mM $NH_4HCO_3$ and twice with 5% formic acid. Peptides were concentrated and de-salted using C-18 ZipTips (Millipore, USA) according to the manufacturer's instructions with the exception of elution solution (60% acetonitrile).

**Mass Spectrometry Identification of Proteins**

Tryptic digestions were mixed 1:1 with α-cyano-4-hydroxycinnamic acid matrix and analyzed with MALDI-TOF-LR-MS (Waters, USA) operating in a positive ion reflectron mode. The mass spectra were externally calibrated with ACTH clip 18-39 (MW 2465.199 Da, Sigma, USA) and internally calibrated with trypsin autolysis peaks (1045.564/2211.108 Da). The peptide mass fingerprints for protein identification were searched automatically at the accuracy of 20-50ppm from UniProt database with ProteinLynx-software (Waters) operating along the instrument. Statistically significantly affected proteins were also searched manually using Matrix Science Mascot Peptide Mass Fingerprint search tool (www.matrixscience.com).

**Western Blotting**

Immediately after the end of the RF-EMF exposure the cells were washed with PBS and harvested with versene. Proteins were extracted with 2% SDS, 1% protease inhibitor cocktail (Sigma, USA). Protein concentrations were measured using Lowry method (Bio-Rad). In total, five replicates were produced. Proteins were separated on 7.5% (GRP78) or 10% (Hsp27, Vimentin) 1D SDS-PAGE and blotted on a PVDF-membrane, blocked with 2% non-fat dry milk, and exposed to primary antibody. The polyclonal Bip (GRP78, Cell Signalling Technology, USA), monoclonal Hsp27 (StressGene, Canada), and vimentin (Zymed, USA) antibodies were used. The respective secondary antibody containing a horseradish peroxidase (HRP)-conjugate (Dako, Denmark) was used. The signal was detected using enhanced chemiluminescence (ECL) (Millipore, USA). Autoradiography films were scanned with GS-710 densitometer (Bio-Rad) and analysed with Phoretix software (Molecular Probes, USA).

## Results and Discussion

In this study we have examined protein expression levels in EA.hy926 cells after the exposure to 1800 MHz GSM mobile phone radiation. Protein expression pattern of EA.hy926 cells was analysed using 2DE with the pH range of 4 - 7 and the gel percentage of 10%, allowing a good separation at the molecular weight (MW) range of approximately 15-150 kDa. In total, 10 replicates were generated from both exposed and sham samples. Such high number of replicates is necessary in order to diminish technical and biological variability, when using silver staining technique to visualize proteins in 2DE gels.

Using PDQuest 7.2 software, about 900 protein spots were detected in the gels. Protein spots, that visually appeared as technical artefacts but were detected by the software, were manually removed from the analysis. Statistical significance of the observed differences in proteins expression levels was determined using student t-test, at the confidence level of 95%, with the assumption of the independent samples. The analysis has revealed eight protein spots which were found to be differentially expressed ($p<0.05$) (Figure 2). Expression of the four of the proteins was found to be down-regulated and four up-regulated by the mobile phone radiation exposure. Down-regulation ratios varied between 0.33-0.47 and up-regulation ratios varied from 1.47 to 2.46.

Comparison of the changes in protein expression pattern observed here and in the earlier study (Nylund and Leszczynski, 2004), shows that exposure to 900 MHz GSM signal has caused expression changes in a larger number of proteins spots and the

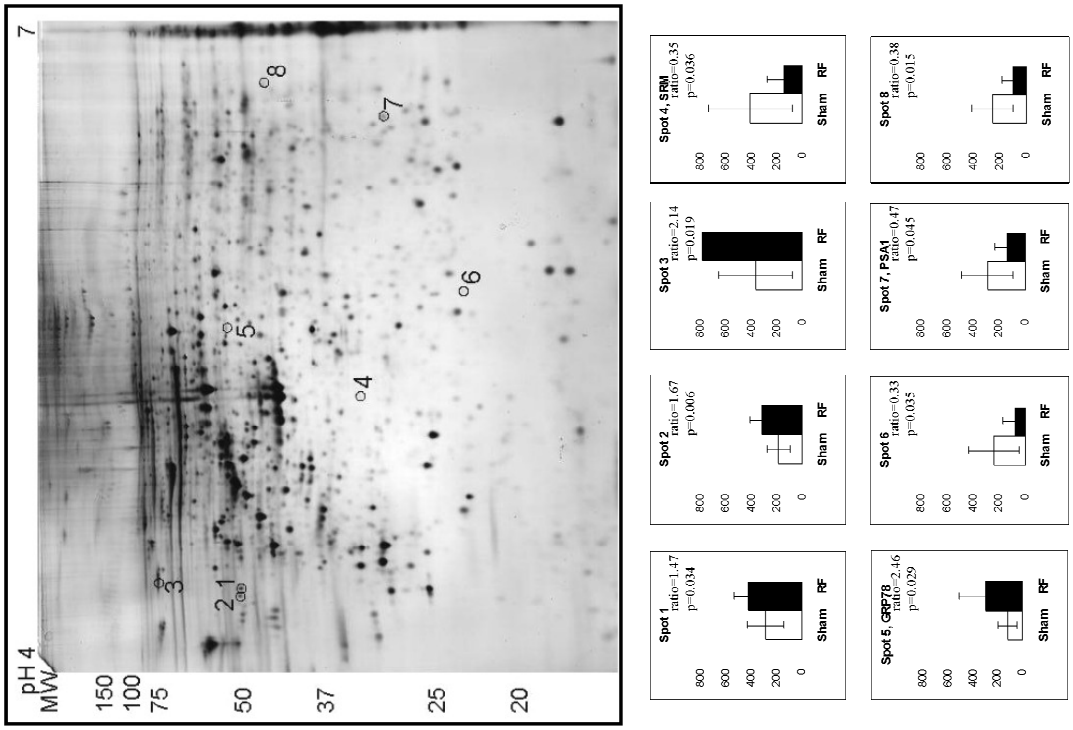


**Figure 2:** A representative gel image showing protein spots with altering expression levels and histograms showing average expression levels and standard deviations of the sham and exposed samples as well as ratio between RF and sham exposed sample (ratio >1 describes up-regulation and ratio <1 down-regulation of the protein). Also t-test p-values are shown.

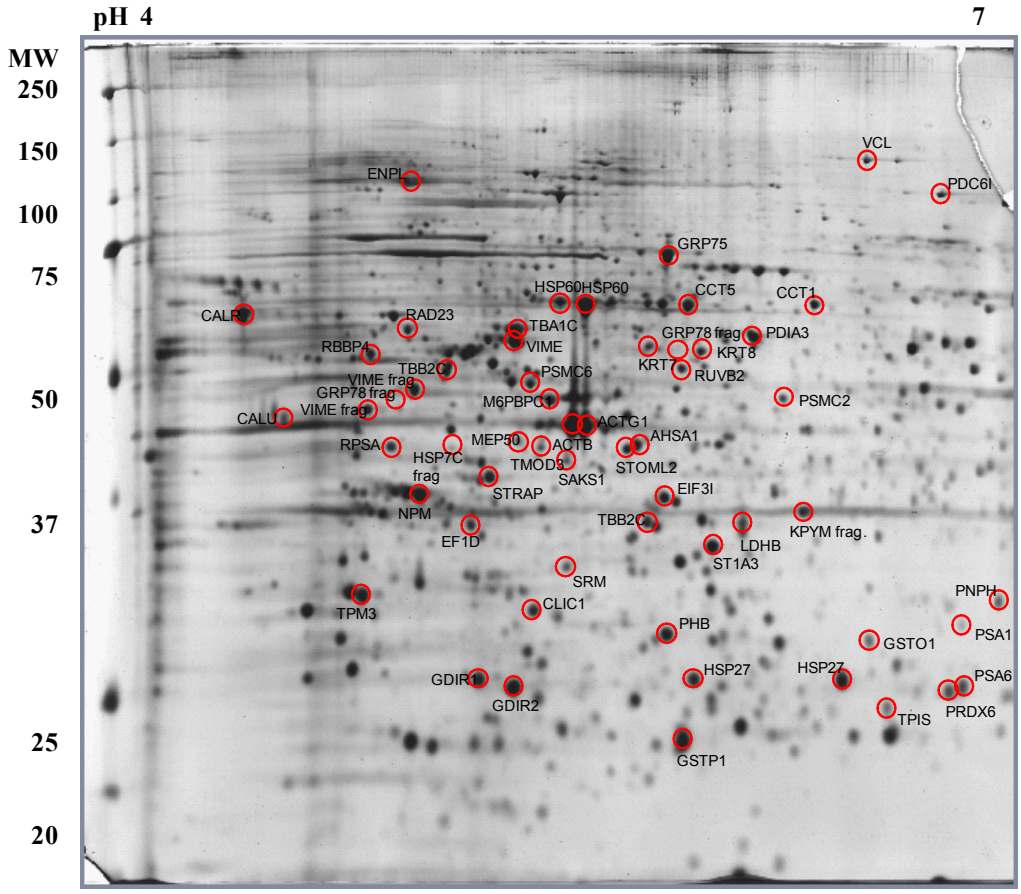


**Figure 3:** A gel image showing the identified protein spots in the EA.hy926 2DE map.

| Gene name | Protein name | Access code | Sequence coverage (%) | MW(kDa)/pI theoretical | MW(kDa)/pI measured |
|---|---|---|---|---|---|
| ACTB | Actin, cytoplasmic 1 | P60709 | 31.5 | 41.7/5.4 | 43/5.4 |
| ACTG1 | Actin, cytoplasmic 2 | P63261 | 40 | 41.8/5.4 | 43/5.5 |
| AHSA1 | Activator of 90 kDa heat shock protein ATPase homolog 1, p38 | O95433 | 72.8 | 38.3/5.5 | 40/5.8 |
| CALR | Calreticulin precursor | P27797 | 49 | 48.1/4.3 | 58/4.3 |
| CALU | Calumenin | O43852 | 45 | 37.1/4.5 | 44/4.5 |
| CLIC1 | Chloride intracellular channel protein 1 | O00299 | 46.5 | 26.9/5.1 | 29/5.3 |
| CCT1/ TCPA | T-complex protein 1 subunit alpha | P17987 | 60.3 | 60.3/6.0 | 60/6.3 |
| CCT5/ TCPE | T-complex protein 1 subunit epsilon | P48643 | 48.2 | 59.6/5.6 | 60/5.9 |
| EF1D | Elongation factor 1-delta | P29692 | 44.8 | 31.1/4.9 | 36/5.2 |
| EIF3I | Eukaryotic translation initiation factor 3 subunit I | Q13347 | 23 | 36.5/5.4 | 37/5.8 |
| ENPL | Endoplasmin precursor | P14625 | 27.5 | 92.4/4.8 | 120/4.9 |
| GDIR1 | Rho GDP-dissociation inhibitor 1 | P52565 | 52.5 | 23.2/5.0 | 26/5.2 |
| GDIR2/ ARHGDIB | Rho GDP-dissociation inhibitor 2 | P52566 | 35.3 | 23.0/5.1 | 25/5.3 |
| GRP75 | Stress-70 protein, mitochondrial (Precursor) | P38646 | 56 | 73.6/6.1 | 74/5.8 |
| GRP78 (frag.) | 78kDa glucose-regulated protein (Precursor) (frag) | P11021 | 26 | 72.4/5.1 | 54/5.9 |
| GRP78 (frag.) | 78kDa glucose-regulated protein (Precursor) (frag) | P11021 | 33.6 | 72.4/5.1 | 48/4.8 |
| GSTO1 | Glutathione transferase omega-1 | P78417 | 45.6 | 27.5/6.6 | 27/6.5 |
| GSTP1 | Glutathione S-transferase P | P09211 | 53.8 | 23.3/5.5 | 23/5.9 |
| HSP27 | Heat shock protein beta-1 | P04792 | 48.3 | 22.8/6.3 | 26/5.9 |
| HSP27 | Heat shock protein beta-1 | P04792 | 37.1 | 22.8/6.3 | 26/6.4 |
| HSP60 | 60 kDa heat shock protein | P10809 | 51 | 61.0/5.8 | 61/5.6 |
| HSP60 | 60 kDa heat shock protein | P10809 | 52.7 | 61.0/5.8 | 61/5.4 |
| HSP7C frag. | Heat shock cognate 71 kDa protein (frag) | P11142 | 25.7 | 71.2/5.4 | 40/5.1 |
| KPYM frag. | Pyruvate kinase isozymes M1/M2 (frag) | P14618 | 46.5 | 58.0/8.2 | 36/6.3 |
| KRT7 | Keratin, type II cytoskeletal 7 | P08729 | 64.8 | 51.4/5.6 | 54/5.8 |
| KRT8 | Keratin, type II cytoskeletal 8 | P05787 | 57.1 | 53.7/5.6 | 54/5.9 |
| LDHB | L-lactate dehydrogenase B chain | P07195 | 46.7 | 36.5/6.0 | 36/6.1 |
| M6PBP1C | mannose-6-phosphate receptor binding protein 1C | O60664 | 64.3 | 47.0/5.4 | 48/5.4 |
| MEP50 | Methylosome protein 50 | Q9BQA1 | 31.6 | 36.7/5.1 | 41/5.3 |
| NPM | Nucleophosmin | P06748 | 44.6 | 32.5/4.7 | 37/4.9 |
| PDCD6IP | Programmed cell death 6-interacting protein | Q8WUM4 | 58.8 | 96.0/6.4 | 105/6.8 |
| PDIA3 | Protein disulfide-isomerase A3 (Precursor) | P30101 | 49.5 | 56.7/6.3 | 56/6.2 |
| PHB | Prohibitin | P35232 | 42.6 | 29.8/5.7 | 28/5.8 |
| PNPH | Purine nucleoside phosphorylase | P00491 | 59.5 | 32.1/6.9 | 30/6.9 |
| PRDX6 | Peroxiredoxin-6 | P30041 | 48.7 | 25.0/6.3 | 25/6.8 |
| PSA1 | Proteasome subunit α type 1 | P25786 | 27 | 29.5/6.6 | 28/6.8 |
| PSA6 | Proteasome subunit α type 6 | P60900 | 52.8 | 27.4/6.7 | 26/6.8 |
| PSMC3/ PRS6A | 26S protease regulatory subunit 6A | P17980 | 85 | 49.2/5.2 | 49/5.3 |
| PSMC2/ PRS7 | 26S protease regulatory subunit 7 | P35998 | 53.6 | 48.6/5.9 | 48/6.2 |
| RPSA | 40S ribosomal prot SA | P08865 | 31.2 | 32.9/4.8 | 40/4.8 |
| RAD23 | UV excision repair protein RAD23 homolog B | P54727 | 27.1 | 43.1/4.8 | 57/4.9 |

| | | | | | |
|---|---|---|---|---|---|
| RBBP4 | Histone-binding protein BBP4 | Q09028 | 31.1 | 47.7/4.8 | 53/4.8 |
| RUVB2 | RuvB-like 2 | Q9Y230 | 69.1 | 51.1/5.6 | 52/5.9 |
| SAKS1 | SAPK substrate protein 1 | Q04323 | 50.5 | 33.3/5.3 | 39/5.4 |
| SRM | Spermidine synthase | P19623 | 19.9 | 33.8/5.4 | 33/5.4 |
| ST1A3 | Sulfotransferase 1A3/1A4 | P50224 | 52.6 | 36.4/5.8 | 35/6.0 |
| STOML2 | Stomatin-like protein 2 | Q9UJZ1 | 46.6 | 38.5/6.9 | 40/5.7 |
| STRAP | Serine-threonine kinase receptor-associated protein | Q9Y3F4 | 56.3 | 38.4/5.0 | 38/5.2 |
| TBA1C | Tubulin alpha-1C chain | Q9BQE3 | 44.5 | 49.9/5.0 | 58/5.3 |
| TBB2C | Tubulin beta-2C chain | P68371 | 50.1 | 49.8/4.8 | 51/5.1 |
| TBB2C (frag) | Tubulin beta-2C chain (frag) | P68371 | 35.3 | 49.8/4.8 | 36/5.8 |
| TMOD3 | Tropomodulin3 | Q9NYL9 | 36.9 | 39.6/5.1 | 40/5.4 |
| TPIS | Triosephosphate isomerase | P60174 | 80 | 26.7/6.9 | 24/6.6 |
| TPM3 | Tropomyosin 3 | Q5VU58 | 69 | 29.2/4.8 | 30/4.8 |
| VIME | Vimentin | P08670 | 78 | 53.6/5.1 | 54/5.3 |
| VIME | Vimentin (fragment) | P08670 | 51.1 | 53.6/5.1 | 49/4.9 |
| VIME | Vimentin (fragment) | P08670 | 66.3 | 53.6/5.1 | 47/4.8 |
| VCL | Vinculin | P18206 | 32.8 | 123.7/5.6 | 130/6.5 |

**Table 1:** All proteins that were identified by MS in EA.hy926 2DE gels.

| spot # | Expression (exposed/sham) | Protein name | Access code | Sequence coverage (%) | Mascot score |
|---|---|---|---|---|---|
| 4 | down | SRM | P19623 | 19.9 | 74 |
| 5 | up | GRP78 fragment | P11021 | 26 | 101 |
| 7 | down | PSA1 | P25786 | 27 | 111 |

**Table 2:** Identified proteins that altered their expression after exposure to 1800 MHz GSM radiation.

changes induced by both exposures were detected in different proteins spots. Previously, using 900 MHz GSM signal, total of 38 protein spots were found to be affected after the mobile phone exposure (Nylund and Leszczynski, 2004), out of which 28 was in the pH range of 4 - 7, as compared with 8 proteins spots that were found here to be statistically significantly affected by 1800 MHz GSM exposure in the same pH range. The number of statistically significantly affected proteins is small (below the number of expected false positives). However, it is possible that some of these proteins might indeed be responding to mobile phone radiation. As shown in our earlier study (Nylund and Leszczynski, 2004), the number of statistically significantly affected proteins might be lower than the expected number of false positives but further analysis using western blot might show that some of the affected proteins (in that particular study - vimentin), might indeed respond to the mobile phone radiation.

Using peptide mass fingerprint (PMF) technique and Maldi-ToF MS, total of 50 protein spots were identified in 2DE gels of EA.hy926 exposed to 1800 MHz GSM mobile phone radiation (Figure 3; Table 1). Among the identified proteins were proteins that we have shown earlier to be affected by 900 MHz GSM radiation: vimentin and Hsp27 (Leszczynski et al., 2002; Nylund and Leszczynski, 2004). Expression of neither of them was altered in a statistically significant manner in 2DE by 1800 MHz GSM radiation (not shown).

Among the 50 identified protein spots were 8 proteins that expression was statistically significantly affected by 1800 MHz GSM radiation. Three of these eight protein spots were successfully identified (Table 2):

- spot #4 - spermidine synthase (P19623 SRM) (Wahlfors et al., 1990), regulates amine and bioamine biosynthesis,
- spot #5 - 78 kDa glucose regulated protein (fragment) (P11021 GRP78) (Ting and Lee, 1988), member of the heat shock protein 70 family, facilitates the assembly of multimeric protein complexes inside the endoplasmic reticulum. The molecular weight of this protein 72.4 kDa, while the affected protein spot observed here was only a fragment of ca. 55 kDa.
- spot #7 - proteasome subunit alpha type 1 (P25786 PSA1) (Silva-Pereira et al., 1992), is a part of large proteasome complex.

Identification of the other five proteins spots with Maldi-ToF was not successful due to low amount of protein in the spots.

Using western blot technique we have attempted to confirm the 2DE results for some of the proteins. Expression changes of GRP78 were examined using polyclonal antibody (Bip/GRP78, Cell Signalling Technology). Two protein bands were detected with MW of 75 kDa (represents the whole protein) and 55 kDa (represents GRP78 fragment identified from our 2DE gels). However, neither of the protein bands appeared to be affected by radiation exposure (Figure 4A). Thus, the western blot technique did not confirm the results obtained with 2DE. Two other identified proteins, SRM and PSA1, were not analyzed using western blot because the corresponding antibodies were not commercially available. Also the western blot experiments for vimentin and Hsp27 have shown a lack of effect of 1800 MHz GSM radiation. For vimentin, using the same antibody as previously (Nylund and Leszczynski, 2004), only a single band was observed in western blot, while in the earlier study the 900 MHz GSM radiation has caused appearance of an additional low-molecular weight vimentin band (Nylund and Leszczynski, 2004). For the single vimentin band observed here there was no

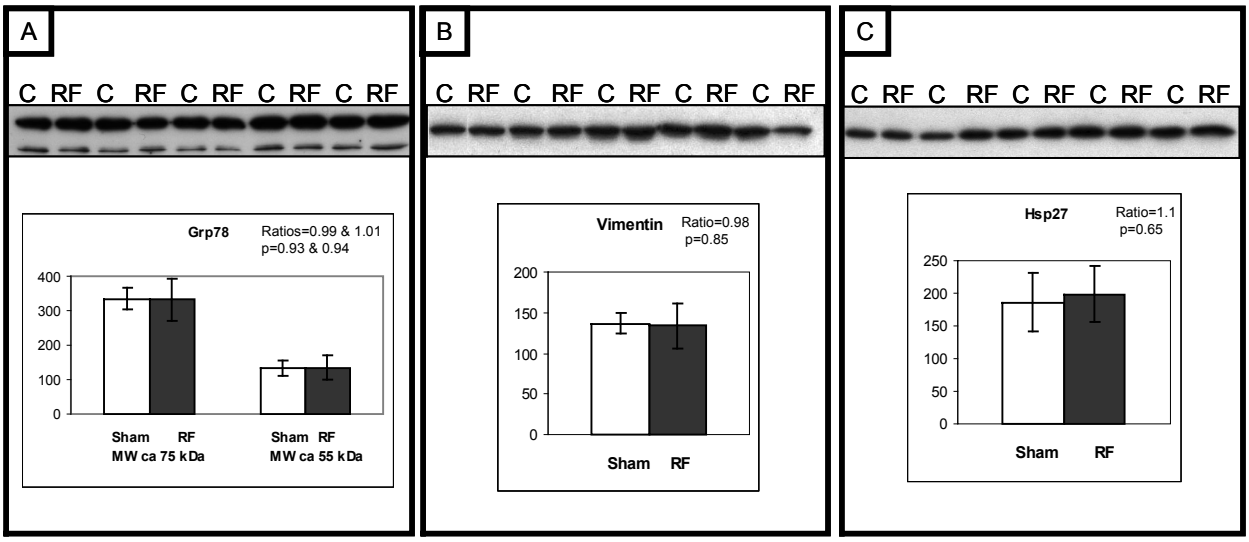


**Figure 4:** Western blots and densitogram bar-graph analyses (mean ± SD) for GRP78 protein (A), vimentin (B) and Hsp27 protein (C). For all western blots EA.hy926 cells were exposed for one hour to 2.0 W/kg 1800 MHz GSM signal using talk-conditions. The experiments were repeated five times. S = sham sample; RF = exposed sample.

change in the expression following the radiation exposure (Figure 4B). For Hsp27, the 2DE gel analyses have shown a statistically non-significant slight increase in the expression but western blot did not show any difference between Hsp27 expression in sham and exposed cells (Figure 4C).

### Future Perspectives

In our previous and in the present study we have used two common mobile phone frequencies, 900 MHz and 1800 MHz, to determine if these radiation frequencies could have any impact on cell proteome. The observed here discrepancy between the responses of EA.hy926 cells to 1800 MHz GSM radiation and the previously published responses of EA.hy926 cells to 900 MHz GSM might be caused either by the different exposure frequencies or by technical differences between the exposure set-ups or by both of the above. The major difference, besides the frequency, between the 900 GSM and 1800 MHz GSM exposure chambers, appears to be the distribution of radiation field within the cell culture dish. In 900 MHz GSM set-up there was non-uniform SAR distribution (Leszczynski et al., 2002). It means that the cells growing in the certain areas of the culture dish were exposed to much higher SAR (over 5.0 W/kg) as compared to the average SAR for the whole cell culture dish (2.4 W/kg) (Leszczynski et al., 2002). In the contrast, the 1800 MHz GSM set-up had very uniform SAR distribution and the cells throughout the cell culture dish were exposed to the same level (2.0 W/kg) of radiation. The possibility of the field-distribution-related effect is supported by our new results showing that stress kinases are activated by the 1800 MHz radiation at 5.0 W/kg but not at 2.0 W/kg (manuscript in preparation). Therefore, there is a need to compare side-by-side the effects of 900 MHz and 1800 MHz frequencies on protein expression and on stress response in EA.hy926 cells using different SAR values.

### Summary Conclusions

Our results suggest that the 900 MHz GSM and 1800 MHz GSM exposures might affect the expression of some proteins in the EA.hy926 cell line. The observed here discrepancy between the expression changes of GRP78 detected with 1DE and 2DE confirms the importance of validation of the results obtained with 2DE using non-high-throughput methods, as e.g. western blot. However, one serious limitation of this approach is the availability of specific antibodies or possession of an animal facility permitting to produce specific antibodies.

### Authors' Contributions

RN developed the proteomics system used here, performed all the analyses presented here, and wrote the draft manuscript. HT performed the 2DE experiments. NK provided the exposure set-up used here. DL obtained the funding of the study and coordinated execution of this project and wrote the final version of the manuscript. All authors have read and approved the final version of the manuscript.

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