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Classification of Biological Effect of 1,763 MHz Radiofrequency Radiation Based on Gene Expression Profiles

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Abstract

Radiofrequency (RF) radiation might induce the transcription of a certain set of genes as other physical stresses like ionizing radiation and UV. To observe transcriptional changes upon RF radiation, we exposed WI-38, human lung fibroblast cell to 1763 MHz of mobile phone RF radiation at 60 W/kg of specific absorption rate (SAR) for 24h with or without heat control. There were no significant changes in cell numbers and morphology after exposure to RF radiation. Using quantitative RT-PCR, we checked the expression of three heat shock protein (HSP) (HSPA1A, HSPA6 and HSP105) and seven stress-related genes (TNFRSF11B, FGF2, TGFB2, ITGA2, BRIP1, EXO1, and MCM10) in RF only and RF/HS groups of RF-exposed cells. The expressions of three heat shock proteins and seven stress-related genes were selectively changed only in RF/HS groups. Based on the expression of ten genes, we could classify thermal and non-thermal effect of RF-exposure, which genes can be used as biomarkers for RF radiation exposure.

Keywords: biomarker, heat shock, quantitative RT-PCR, radiofrequency radiation

Introduction

Non-ionizing radiofrequency (RF) radiation refers to the electromagnetic waves ranging between 10 MHz and 300 GHz. It is constantly produced by many electronic devices sources such as mobile phones, base stations, television and computer. We are surrounded by these electronic devices in daily living and working environments. Because the usage of radiofrequency radiation is persistently increasing, we need to understand its biological effect at cellular and molecular levels in detail.

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HSPs have been well known as a marker of impending cell damage and indicator of cellular stress such as heat shock as well as RF radiation (Tian *et al*., 2002; Yu *et al*., 2007). When biological samples is exposed to RF radiation at a low SAR (<1 W/kg) or for short duration, the temperature remains constant within a normal physiological range. In contrast, if we apply higher SAR (> 10 W/kg) and/or prolonged RF exposure, the temperature of exposure system is increased even in the presence of cooling system. In this sense, the expression of HSP70 increased time- and dose-dependently at above 50 W/kg SAR of 2,450 MHz for 1~3 h in glioblastoma cells (Wang *et al*., 2006). However, the expression of heat shock proteins may change upon non-specific stimuli.

Recent studies have focused on effect of non-thermal RF radiation in biological systems (Chauhan *et al*., 2007; Huang *et al*., 2008; Lee *et al*., 2006; Luukkonen *et al*., 2009; Qutob *et al*., 2006; Sanchez *et al*., 2007; Takashima *et al*., 2006; Verschaeve, 2009). Most of these studies have shown no significant effect on cellular and molecular levels in a variety of condition. Microarray analysis of gene expression showed that there is no evidence that non-thermal RF radiation affect gene expression in U87MG glioblastoma cells exposed to 1.9 GHz RF at SARs from 0.1 to 10 W/kg for 4 h and 24h, respectively (Chauhan *et al*., 2007; Qutob *et al*., 2006). Takashima *et al*. (2006) also failed to find any changes in cell proliferation with continuous exposure at up to 100 W/kg except at SAR of 200 W/kg suggesting that RF radiation at 200 W/kg SAR affected the cells by combined with heating medium. Similarly, Sanchez *et al*. reported that there were no effects of 1,800 MHz RF radiation at 2 W/kg SAR on HSP expression and apoptosis in human skin cells compare to ultraviolet (UV) and heat shock (Sanchez *et al*., 2007). In our previous studies, neither of cell cycle changes nor DNA damage as was detected in after 1,763 MHz RF exposure at an SAR of 10 W/kg SAR in Jurkat-T cells or 20 W/kg in auditory hair cells (Huang *et al*., 2008).

Although most of the studies on the biological effect of RF exposure could not detect any molecular changes, some reports could demonstrate RF-specific gene expression. Exposure of 2.45 GHz high frequency electromagnetic field to glioma cells at SAR levels above 20 W/kg led to an increased level of HSP70, even when the effect of raised temperature is taken into account (Tian *et al*., 2002). Lee *et al*. found that 2.45 GHz RF

radiation at 10 W/kg of SAR for 2～6 h affected expression of 221 genes including apoptosis-related genes and the cell cycle genes in human HL60 cells without significant increase of HSP expression (Lee *et al*., 2005). 1,800 MHz RF radiation at 2 W/kg got 24 h in rat neuron altered expression of cytoskeleton genes such as microtubule-associated protein 2 (MAP2) and signal transduction-related genes as well as immediate early gene (EGR1) (Zhao *et al*., 2007). Recently, it has been shown that exposure to 900 MHz GSM-modulated RF at 1 W/kg SAR for up to 72 h alters morphological maturation of neural cells through cytoskeleton regulating factors such as β-thymosin (Del *et al*., 2009). These reports suggest that RF exposure might alter the cytoskeleton and activates signal transduction pathways, thus lead to the abnormal neural growth. Yu *et al*. also found that RF exposure induce HSP27 and HSP70 as well as phosphorylation of ERK1/2 and JNK1/2 in human lens epithelial cells (Yu *et al*., 2007).

In this study, we exposed normal human fibroblast, WI-38 cells to 1,763 MHz RF radiation at 60 W/kg SAR for 24h and evaluated effects of RF radiation on molecular level by quantitative RT-PCR for three HSP genes and seven stress-related genes, which were selected from gene expression analysis using microarray on RF-exposed cells.

Methods

Cell culture

Normal human lung fibroblast, WI-38 cells were purchased from ATCC and grown in DMEM (Wellgene, Korea) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 mg/ml streptomycin, and 100 U/ml penicillin (Invitrogen, Carlsbad, CA, USA) at 37°C in a 5% CO_2 incubator. The cells with passage number from 18 to 21 were used for this study. Morphological changes in cells were observed under phase contrast microscope (magnification 40X) and cell numbers were checked using hemocytometer.

Radiofrequency (RF) radiation exposure system

The condition for RF exposure was described in the previous study (Lee *et al*., 2006). Briefly, a real Code Domain Multiple Access (CDMA) signal at 1,762.5 MHz was applied for RF exposure. The exposure system was first equilibrated to 37°C for 2 h and WI-38 cells in 100 mm culture dish containing 18 ml of growth medium were exposed at SAR values of 60 W per kg for 24 h. The temperature in the chamber was maintained at 37±0.2°C by circulating water within the cavity for "RF only" samples (n=4), while the temperature in the chamber was not controlled for "RF/HS" samples (n=4). Before and after RF radiation exposure, temperature of each sample was measured (Fig. 1). Cells were immediately harvested, counted and processed for further analysis. Cells were also divided separately and cultured at 37°C in a 5% CO_2 incubator as a control (n=8).

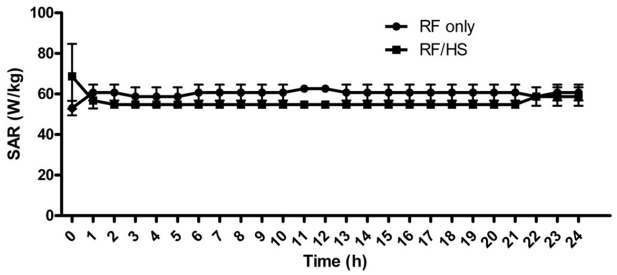

Fig. 1. Monitoring SAR values for 1,763 MHz RF radiation. WI-38 human normal lung fibroblasts were exposed to 1,763 MHz RF radiation for 24 hours and the output in exposure chamber were shown in graphs recorded for 24 h of RF radiation exposure.

Table 1. List of primers for quantitative RT-PCR

Gene	RefSeq	Forward	Reverse
BRIP1	NM_032043.1	CGCCACAGAAACCCCCTGGC	GCCGTCCTCCGGAGCTCTCT
EXO1	NM_006027.3	TGCCAGAGCCAGTGGGCTGA	TGGGGACAGGGGTTTCTTACAAGG
MCM10	NM_182751.1	AGGGTCCTCCCTGCTCCTGC	GCCGGGGAGACTTCTCTACAGGG
TGFB2	NM_003238.2	CCCCTCCGAAAATGCCATCCCG	TCCGTTGTTCAGGCACTCTGGC
TNFRSF11B	NM_002546.3	GTGCTGCGCGCTCGTGTTTC	CAAGGGGCGCACACGGTCTT
FGF2	NM_002006.4	GCGACCCTCACATCAAGCTA	GCCAGGTAACGGTTAGCACA
HSP105	NM_006644.2	CGAATTCCAGCTGTGAAGGA	AACTTTAAATGCCGGGGAAA
HSPA6	NM_002155.3	GACAGAGTGGCTGCCAAAAA	TCCCGACACTTGTCTTGCAT
ITGA2	NM_002203.3	GCGACGAAGTGCTACGAAAG	CCCAAGAACTGCTATGCCAA
HSPA1A	NM_005345.5	ACAAGTCCGAGAACGTGCAG	CGTACACCTGGATCAGCACC
GAPDH	NM_002046.3	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC

Quantitative RT-PCR

Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. One microgram of total RNA was converted to cDNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo-$(dT)_{12-18}$ primers (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The qRT-PCR was performed in a total reaction mixture containing cDNA, SYBR Premix Ex Taq (Takara Bio Inc., Shiga, Japan), Rox reference dye, and primers specific for each gene (Table 1). Samples were analyzed with the ABI PRISM 7000 sequence detection system (Applied BioSystems, Foster City, CA). All PCRs were performed in triplicate, and the specificity of the reaction was determined by melting curve analysis at the dissociation stage. We normalized each value with GAPDH and represented relative expression to control.

Hierarchical clustering

Hierarchical clustering analysis was performed with the standardized expression values using the "Manhattan" distance metric, and the "Ward" linkage algorithm. The expression values were standardized across samples with the median value and inter-quartile range (IQR) of each transcript. Statistical supports for the clustering were obtained by bootstrapping (BP) analysis and the approximately unbiased (AU) test using the "pvclust" package of R statistical software. For the informativeness of genes, we used the signal-to-noise ratio as previously described (Furey *et al*., 2000).

Statistical analysis

Each value measured in independent experiments was represented as the mean±standard deviation (SD).

Results and Discussion

To understand the non-thermal effect of RF radiation, we exposed WI-38, normal human lung fibroblast cells to 1,763 MHz RF at a SAR of 60 W/kg for 24 hours with (RF only) or without (RF/HS) operating cooling system and checked the expression of genes by quantitative RT-PCR. We recorded output of RF exposure system, and also measured temperature of medium for eight samples right after RF exposure (Fig. 1). Even though there are some variations in SAR, RF radiation was almost constantly maintained for 24 hours. We had four samples for RF only group with controlling temperatures at 37°C during RF exposure and the other four samples for RF/HS group were exposed to RF radiation and heat at 40～42°C. Among these two groups, we could not find any significant changes in morphology and number of cells in comparison to unexposed control cells (Fig. 2A). On the other hand, 24 hr-heat shock exposure resulted in the decrease of cell numbers to 50-70% (Fig. 2B). Long term heat shock exposure might

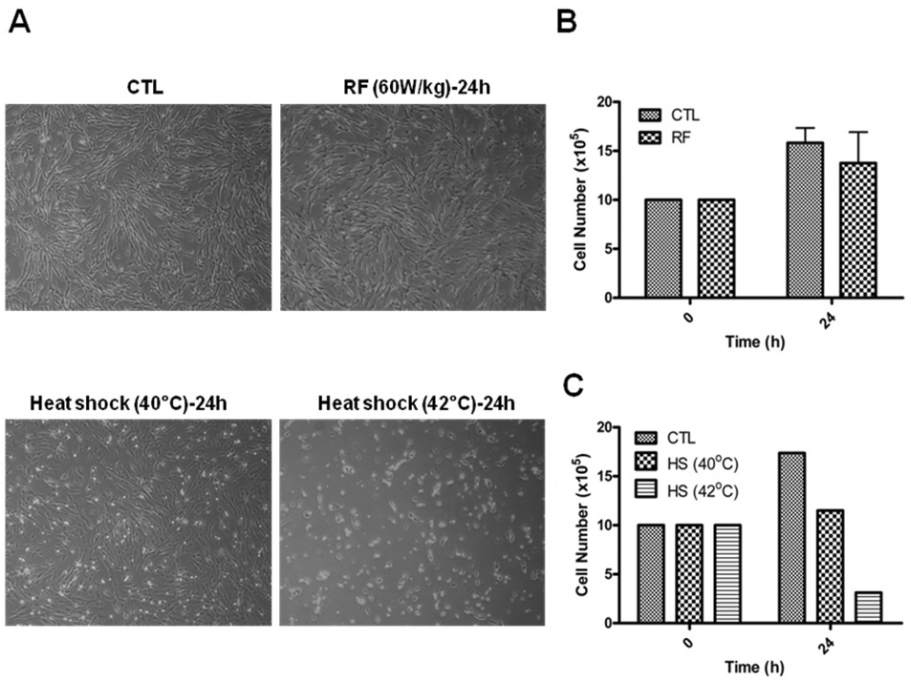

Fig. 2. Cell morphology (A) and cell numbers (B, C) after RF and heat shock exposure.

induce cell death, while RF radiation with heat shock for the same duration in RF/HS group did not induce cell death.

HSPs have been widely used as a marker of impending cell damage and indicator of cellular stress such as heat shock as well as RF radiation (Tian *et al.*, 2002). We measured the level of mRNAs for HSPA6, HSP1A1 and HSP105 using quantitative RT-PCR in two groups. As shown in Fig. 3A, the expression levels of three HSPs in RF/HS groups and heat shock control group were higher than RF only exposure and control samples. Taken together, the expression of HSPs tested in this study were consistently elevated in RF/HS exposure samples, but not in RF only groups. Even the exposure at higher SAR (60 W/kg) and longer duration did not induce HSPs as it did at a lower SAR (10～20 W/kg) in previous results (Huang *et al.*, 2008). We tried to separate the non-thermal effect of RF radiation using intensive temperature control system to observe the expressions of HSP. Because hot spots might occur within

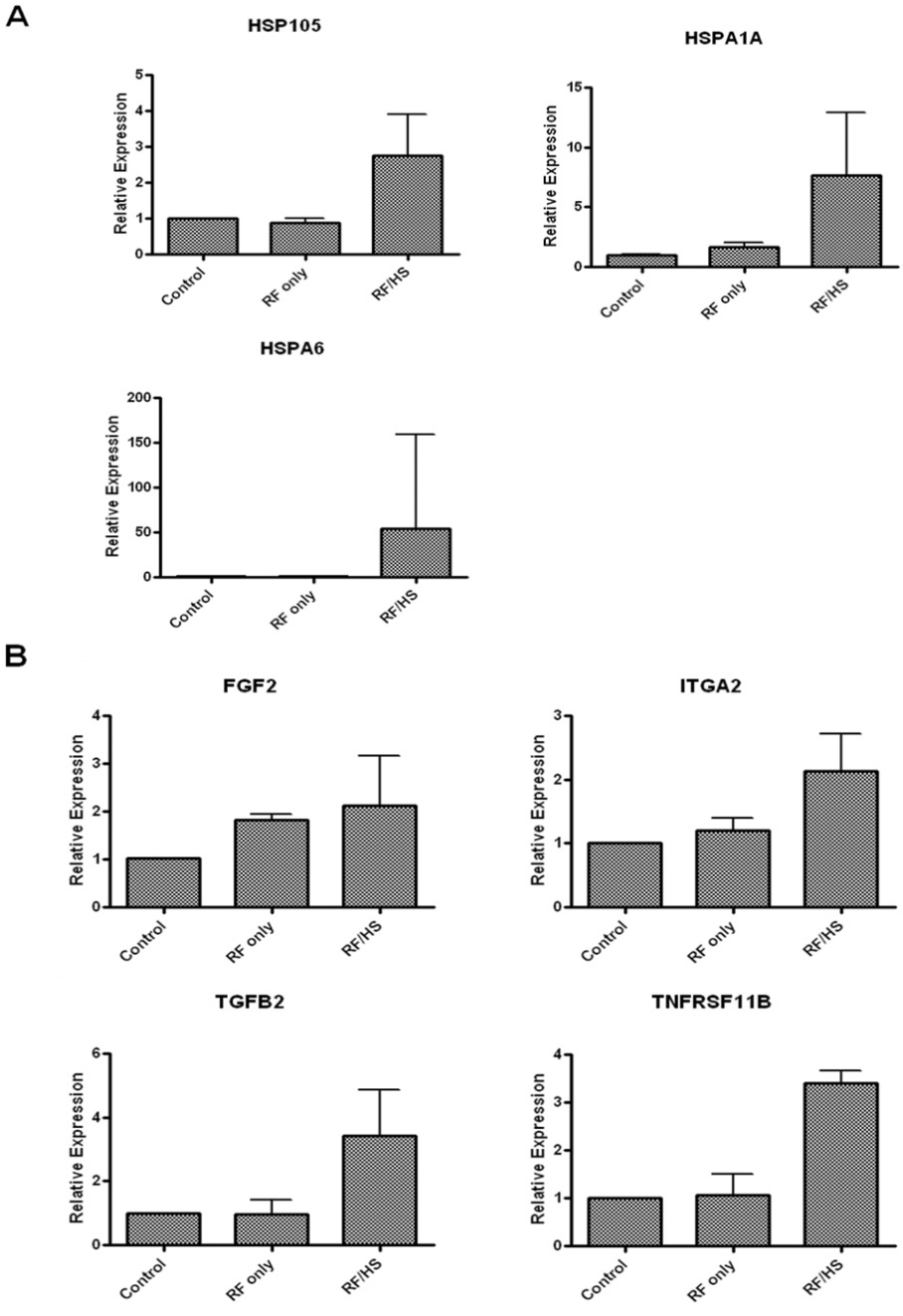

Fig. 3. Quantitative RT-PCR against representative HSPs (A), up-regulated genes (B) and down-regulated genes (C).

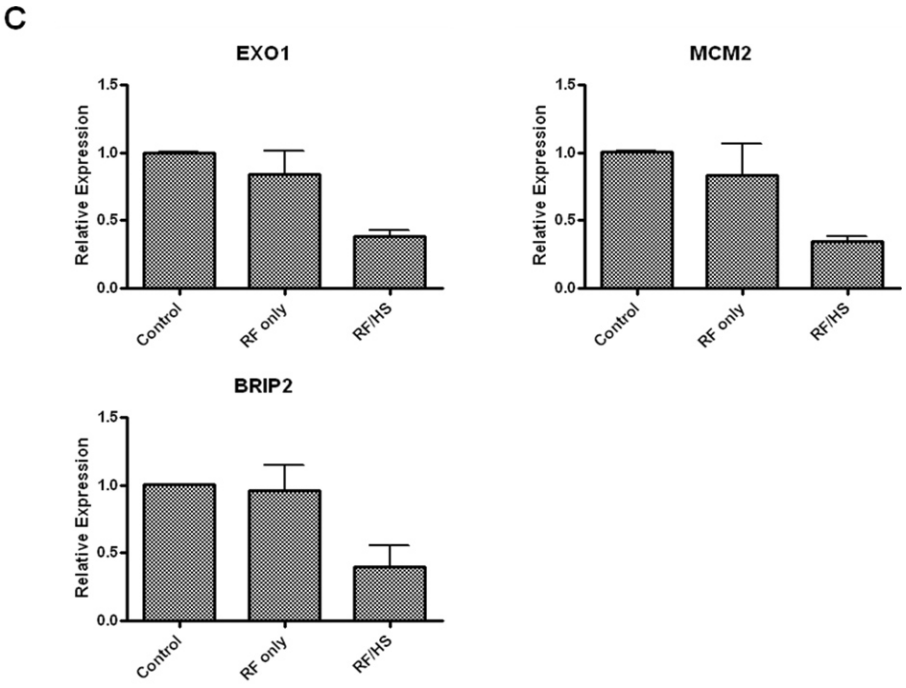

Fig. 3. Continued.

cells in exposure chamber, RF radiation energy might be not deposited on biological sample in a homogeneous manner (McNamee *et al.*, 2009). In addition, Tian *et al.* (2002) suggested that high frequency electromagnetic field exposure can induce HSP70 expression through an unknown mechanism other than a thermal effect. From our results, we suggest that heat shock stimuli (40 or 42°C) might be distinct from thermal RF radiation. It can be intriguing to assess whether there are the differences between heat shock and thermal RF radiation in respect to molecular and cellular levels.

From our microarray datasets using various cell lines on stress responses, we selected seven stress-related genes which significantly were up-regulated (TNFRSF11B, FGF2, TGFB2, and ITGA2) or down-regulated (BRIP1, EXO1, and MCM10) in RF radiation exposure compared to unexposed control. We checked the expressions of seven genes in RF only, RF/HS and control samples using quantitative RT-PCR (Fig. 3). Although expression levels of genes tested in this study highly varied among samples, mRNA levels of TNFRSF11B ($p < 0.05$), FGF2 ($p < 0.01$) and ITGA2 ($p < 0.05$) were significantly up-regulated only in RF/HS groups. The expression levels of BRIP1 ($p < 0.05$), EXO1 ($p < 0.01$), and MCM10 ($p < 0.01$) were significantly down-regulated only in RF/HS samples. None of seven genes were changed in RF only group in comparison to the control group.

From the expression profiling data of ten genes, we tried to check whether eight RF exposure samples could be classified. Using the normalized expression values

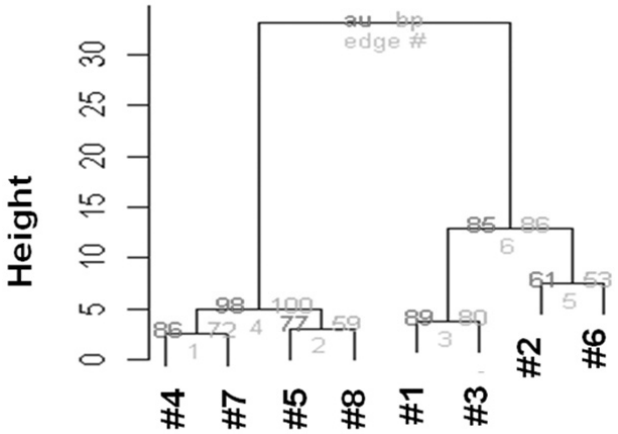

Fig. 4. Hierarchical clustering analysis on eight independent samples for RF exposure.

from quantitative RT-PCR for tested ten genes, we applied hierarchical clustering method to analyze the expression patterns. Statistical supports for it were obtained by bootstrapping (BP) analysis and the approximately unbiased (AU) test. As shown in Fig. 4, cluster dendrogram with AU/BP values (%) indicated that four RF radiation samples of RF/HS group are closely clustered while other four samples of RF only group gathered to one cluster. Among ten genes, two HSPs (HSPA1A, HSP105), two up-regulated genes (TNFRSF11B, TGFB2) and two down-regulated genes (EXO1, MCM10) are informative to understand that these are affected by RF

Table 2. Informativeness of genes tested by quantitative RT-PCR

Gene	−LOG10 (p value)	Signal to noise ratio
HSPA6	0.75	0.28
HSP105	1.41	1.46
HSPA1A	3.95	1.06
FGF2	0.65	0.26
ITGA2	0.74	1.18
MCM10	0.41	1.79
TGFB2	1.98	1.29
TNFRSF11B	2.21	3.38
EXO1	0.38	2.13
BRIP1	0.48	1.65

radiation (Table 2).

Four up-regulated genes are related to the cellular communication. For instance, TNFRSF11B (also known as osteoprotegerin, OPG) is a member of the TNF-receptor superfamily and reported to inhibit osteoclast formation and function. This gene is specifically stimulated by TGFB including TGFB2 and TGFB3 (Thirunavukkarasu *et al*., 2001). Considering that both TNFRSF11B and TGFB2 were up-regulated by RF radiation, effect of RF radiation at high SAR on osteoclast differentiation may be interesting. Meanwhile, down-regulated four genes are related to DNA metabolism. For instance, EXO1 encodes a protein with 5' to 3' exonuclease activity as well as an RNase H activity. It is similar to the Saccharomyces cerevisiae protein Exo1 which is involved in mismatch repair and recombination (Genschel *et al*., 2002). BRIP1 (as known BACH1) is a member of the RecQ DEAH helicase family and directly interacts with the C-terminal BRCT repeat of the breast cancer susceptibility protein BRCA1 (Kumaraswamy *et al*., 2007). This report suggests that BRIP1 is critical not only for the timely progression through the S phase but also for maintaining genomic stability. We previously could not find any changes of cell cycle and DNA damage after exposure of 1763 MHz at 10 or 20 W/kg SAR for up to 48h in auditory hair cells and Jurkat-T cells, respectively (Huang *et al*., 2008). This discrepancy of our observations may result from difference in cell type and RF radiation dosage. In respect with cell cycle, further study for evaluation of these genes is required after RF radiation exposure at high SARs.

Many studies indicate that biological effect of RF radiation is controversial or even not reproducible. Some reasons including different exposure system, thermal confounding factor, cell type and experimental variations can be hypothesized for these different observations although it still remains unclear. Therefore, we could not exclude the possibility that RF exposure affect biological system on cellular and DNA level. Most important, there are no reliable indicators for RF radiation on molecular level. We applied 1,762.5 MHz RF radiation at 60 W/kg of SAR to normal fibroblast, WI-38 cells for 24 h. We failed to find significant changes in cellular proliferation and morphology after RF radiation exposure compared to heat shock treatment. However, analysis of quantitative RT-PCR for HSPs showed high variations in expression levels among RF radiation exposed samples. In addition, RF radiation affected expression levels of genes including TNFRSF11B, EXO1, and MCM10. Since it has been unknown about relationship between above genes and RF radiation, they may be potential indicators to differentiate thermal effect and non-thermal effect during RF radiation. It will be interesting to examine whether expression levels of these genes after RF radiation exposure are distinct from that of other stressors such as UV, ionizing radiation in further study.

Acknowledgments

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