

## RESEARCH ARTICLE

# Analysis of gene expression in two human-derived cell lines exposed *in vitro* to a 1.9 GHz pulse-modulated radiofrequency field

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There is considerable controversy surrounding the biological effects of radiofrequency (RF) fields, as emitted by mobile phones. Previous work from our laboratory has shown no effect related to the exposure of 1.9 GHz pulse-modulated RF fields on the expression of 22 000 genes in a human glioblastoma-derived cell-line (U87MG) at 6 h following a 4 h RF field exposure period. As a follow-up to this study, we have now examined the effect of RF field exposure on the possible expression of late onset genes in U87MG cells after a 24 h RF exposure period. In addition, a human monocyte-derived cell-line (Mono-Mac-6, MM6) was exposed to intermittent (5 min ON, 10 min OFF) RF fields for 6 h and then gene expression was assessed immediately after exposure and at 18 h postexposure. Both cell lines were exposed to 1.9 GHz pulse-modulated RF fields for 6 or 24 h at specific absorption rates (SARs) of 0.1–10.0 W/kg. In support of our previous results, we found no evidence that nonthermal RF field exposure could alter gene expression in either cultured U87MG or MM6 cells, relative to nonirradiated control groups. However, exposure of both cell-lines to heat-shock conditions (43°C for 1 h) caused an alteration in the expression of a number of well-characterized heat-shock proteins.

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**Abbreviations:** **CT**, comparative threshold; **CW**, continuous wave; **MM6**, Mono-Mac-6; **qRT-PCR**, quantitative real-time PCR; **RF**, radiofrequency; **SAR**, specific absorption rate; **VSN**, variance-stabilization and normalization; **W-CDMA**, wideband-code division multiple access

## 1 Introduction

Increasing usage of cellular phones and their growing presence in our lives have aroused much public concern as to potential health risks associated with the radiofrequency (RF) fields emitted by these devices. Reports of associations between RF fields and cancer rates have created widespread concern among the public and prompted researchers to examine further the biological effects associated with exposure to RF fields [1]. Although the majority of the data pub-

lished to date has not provided convincing evidence of adverse effects related to nonthermal RF field exposure [2–4], a limited number of studies have reported contrary findings [5–9]. Clearly, it remains critical to resolve discrepancies and to identify any potentially negative biological changes that result from RF field exposure in order to prevent human health consequences before they arise.

Several recent studies have explored changes in gene expression as a basis for understanding the molecular changes that may occur following RF field exposure. A number of investigations monitored the expression levels of heat-shock proteins (HSP) and proto-oncogenes in a variety of cell systems and reported altered expression of these genes following RF field exposure [5–9]. However, other studies failed to detect such effects following similar RF field exposure conditions [10–13]. Recently, Lantow *et al.* [14] exposed human-derived Mono-Mac-6 (MM6) and K562 cells to 1.8 GHz continuous wave (CW) and global system for mobile communications (GSM)-modulated RF fields at specific absorption rates (SARs) of 0.5–2.0 W/kg and found no significant effects in HSP70 expression in either cell-line at any of the RF field exposure levels tested [14]. Previous studies in our laboratory also showed no induction in mRNA expression of HSP27, HSP70 and the proto-oncogenes c-jun, c-fos, and c-myc following exposure of TK6, MM6, and HL-60 cells to intermittent (5 min on, 10 min off) 1.9 GHz pulse-modulated RF fields at average SARs of 1 and 10 W/kg for 6 h and at 18 h postexposure [15, 16].

With the emergence of high throughput screening technologies, it has become feasible to analyze the expression of thousands of genes in parallel. However, while this technology is being more widely applied in biological research it can only be exploited through the appropriate experimental design and interpretation of data. In an early study, Harvey and French [17] reported that exposure of human mast cells (HMC-1) to 864.3 MHz CW RF fields at a SAR of 7 W/kg for  $3 \times 20$  min/day for 7 days, altered the localization of protein kinase C and caused differential expression of three genes from a 588 gene cDNA microarray. The affected genes included the proto-oncogene c-kit, the transcription factor Nucleoside diphosphate kinase B, and the apoptosis-associated gene DAD-1. Lee *et al.* [18] found differential expression of over 750 genes using SAGE in HL-60 cells exposed to 2.45 GHz pulse-modulated RF fields for 6 h at a SAR of 10 W/kg. More recently, Remondini *et al.* reported that mobile phone radiation induced differential gene expression of 12–34 genes in three of six human-derived cell lines exposed to 900 and 1800 MHz mobile phone radiation. The authors concluded that some human cell-lines may be more sensitive to mobile phone radiation than others [19].

In contrast to the above studies, Hirose *et al.* [20] evaluated markers of apoptosis and stress in two human cell lines following RF field exposure. Human glioblastoma A172 cells were exposed to 2.1425 GHz wideband-code division multiple access (W-CDMA)-modulated RF fields at SARs of 80–800 mW/kg for 24 h, while human-derived IMR-90

fibroblasts from fetal lungs were exposed to 2.1425 GHz CW and W-CDMA-modulated RF fields at a SAR of 80 mW/kg for 28 h. No significant changes in expression of phosphorylated p53 or total p53 were found from the RF field exposure. Furthermore, neither microarray nor quantitative real-time PCR (qRT-PCR) analysis showed any noticeable differences in gene expression of the subsequent downstream targets of p53 signaling in the RF field exposed groups relative to the sham controls. Zeng *et al.* [21] also found no convincing changes in gene or protein expression in MCF-7 cells exposed to 1.8 GHz RF field at a SAR of up to 3.5 W/kg for 24 h. Although a small number of gene expression changes were detected using DNA microarrays, this differential expression could not be confirmed by qRT-PCR. Previously our laboratory also examined the effect of 1.9 GHz pulse-modulated RF fields on changes in the expression of 22 000 genes in human glioblastoma-derived cells at 6 h following a 4 h exposure. Results from this study showed no evidence of differential gene expression in the RF field exposed samples relative to the sham control [22].

Therefore, despite extensive research, the evidence on the potential health consequences of RF fields remains controversial and further research in this area is needed. In this study, we extend our previous investigations of RF-induced gene expression, by exploring for late on-set genes in U87MG cells at 6 h following a 24 h exposure to continuous 1.9 GHz pulse-modulated RF fields. We have also assessed whether exposure to intermittent (5 min ON, 10 min OFF) RF fields could influence gene expression in human-derived (MM6) cells. The U87MG and MM6 cell lines were chosen based on the fact that they have both been extensively used in gene expression and RF field bioeffects studies and are well characterized.

## 2 Materials and methods

### 2.1 U87MG cell cultures

Human glioblastoma-derived U87MG cells were obtained from American Type Culture Collection (ATCC; Manassas, VA) and cultured in MEM- $\alpha$  media containing 2 mM of L-glutamine, supplemented with 10% FBS (Sigma-Aldrich; St. Louis, MO), and antibiotics (100 U/mL penicillin, 100  $\mu$ g/mL streptomycin; Invitrogen, Burlington, ON), in a standard tissue culture incubator (5% CO<sub>2</sub>, 37°C). Cell concentrations were maintained below  $1 \times 10^6$  cells/mL by subculturing every 2–3 days. Two days prior to experimentation, cells were subcultured to a concentration of  $1 \times 10^5$  cells/mL in 60 mm culture dishes. On the day of experimentation, the cultures were approximately 70% confluent when they were exposed to sham, RF, positive (heat-shock) control, or negative (incubator) control conditions. Following exposure, cultures were returned to a standard tissue culture incubator. Cell viability was ascertained in each of the samples immediately prior to

and following the RF field exposure period and at 6 h post-exposure, using the dual stain viability assay [23].

## 2.2 MM6 cell cultures

MM6 cells (DSMZ; Mascheroder, Germany) were grown in RPMI 1640 media supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cell concentrations were maintained below  $1 \times 10^6$  cells/mL by subculturing every 2–3 days. Cells were seeded 1 day prior to RF exposure to attain a cell concentration of approximately  $1.5\text{--}2.0 \times 10^5$  cells/mL at the beginning of each experiment ( $T = 0$  h). Ten milliliters of cell culture was transferred into 60 mm culture dishes and placed into the RF exposure chamber. Cell viability was determined, as mentioned above.

## 2.3 RF exposure conditions

Six circularly polarized cylindrical waveguides were used to expose the cell culture samples to 1.9 GHz pulse-modulated (50 Hz, 1/3 duty cycle) RF fields. U87MG cells were exposed for 24 h at mean SARs of 0 (sham), 0.1, 1.0, and 10.0 W/kg, while MM6 cells were exposed for 6 h at mean SARs of 0 (sham), 1.0, and 10.0 W/kg. Within the sample region, the maximum to minimum SAR ratio was approximately 4:1 [24], while the SAR distribution in the plane of the cells was estimated to be  $\pm 24\%$  of the mean SAR established for each RF treatment group.

Temperature within the cell cultures was monitored at 60 s intervals during RF exposure using nonperturbing thermistor probes, as previously described [22]. Both negative (incubator) and positive (heat-shock) controls were run in conjunction with each of the five independent experiments. The positive control cultures were subjected to an elevated temperature of 43°C for 1 h using a heating block placed within an incubator. Heat-shock conditions were not applied to the negative control samples; however, the negative control cultures were housed in the same incubator for the same 1 h time period. Confirmation of the temperatures within the positive (heat-shock) and negative control samples was obtained using sterile thermocouple probes in direct contact with the culture medium. For U87MG cells, heat-shock conditions were applied for 1 h at  $T = 23$  h, six hours prior to harvesting the  $T = 24$  h samples. For MM6 cells, heat-shock conditions were applied for 1 h at  $T = 0$  h and RNA was isolated at 6 and 24 h later. Following heat-shock and/or negative control conditions, the control samples were cultured in a manner akin to those applied to RF-exposed samples.

## 2.4 RNA extraction, amplification, and hybridization of U87MG cells

A 350 µL aliquot of guanidine isothiocyanate (GITC) containing lysis buffer (RNeasy Mini kit; Qiagen, Mississauga, ON) was applied directly to cell monolayers (U87MG cells) or

cell pellets (MM6 cells) in order to lyse the cells. The samples containing the lysis buffer were subsequently frozen at  $-80^\circ\text{C}$  and processed at a later date. The lysate collected from each sample was then applied to a QIAshredder spin column and placed in 2 mL collection tubes (Qiagen) following thorough mixing by pipetting. After a 2 min ( $\sim 13\,000$  rpm) centrifugation and the subsequent addition of 70% ethanol, the solution was mixed by pipetting to precipitate the RNA. RNeasy Mini Kits and On-Column RNase-free DNase (Qiagen) were used as *per* the manufacturer's instructions (Qiagen) to extract the RNA. An Agilent 2100 Bioanalyzer along with RNA Nanochips (Agilent Technologies Canada; Mississauga, ON) and an Ultraspec spectrophotometer ( $A^{260}$ : $A^{280}$ ) were used to assess RNA quality and concentration for each sample. All samples included for analysis were determined to be of high quality RNA ( $\text{OD}_{260/280} = 1.8\text{--}2.1$ ).

Low RNA Input Fluorescent Linear Amplification Kits (Agilent Technologies Canada) were used to generate fluorescently labeled cRNA. Briefly, MMLV-Reverse transcriptase and dT-T7 primer were used to amplify mRNA from total RNA samples into dsDNA. Cyanine 3-labeled cRNA was produced using T7 polymerase. The labeled cRNA was purified using RNeasy Mini kits and concentration was measured using a spectrophotometer. cRNA (1 µg) was fragmented for 30 min at 60°C and subsequently hybridized to Agilent Human 1A (V2) oligonucleotide 22K microarray slides (Agilent Technologies Canada) in stainless steel hybridization chambers. Hybridization was carried out over 17 h in the dark in a rotating hybridization chamber set at 60°C and 4 rpm. Slides were washed using  $6 \times$  SSPE solution (Amresco, Solon, Ohio, USA) containing 0.005% *N*-laurylsarcosine (Sigma-Aldrich) for 1 min followed by a 1 min wash with  $0.06 \times$  SSPE containing 0.005% *N*-laurylsarcosine and a final 30 s wash with Agilent Stabilization and Drying solution (Agilent Technologies Canada).

## 2.5 Semiquantitative RT-PCR analysis

MuLV-Reverse transcriptase was used to generate single-stranded cDNA from total RNA extracted from U87MG cells. In short, each reaction mixture of 1 µg RNA, 25 mM  $\text{MgCl}_2$ , 10 mM of each dNTP, 20 U/mL of RNase inhibitor and 50 µM of random hexamers was incubated at 45°C for 60 min and was followed by denaturation at 99°C for 5 min. qRT-PCR reactions were carried out using 2.2 µL of the cDNA generated, 200 nM of gene specific primers, and SYBR green supermix (BioRad; Hercules, CA). The determined target gene sequences served as the basis for gene specific primers designed using Primer3 software. Primer sequences were previously published [16, 22]. Reactions were run in duplicate in a spectrofluorometric thermal cycler (Biorad iCycler; Hercules, CA) and prepared in 96-well plates. Normalization was achieved using  $\beta$ -actin mRNA as an endogenous control and all primer sets were analyzed with RT  $\pm$  cDNA. Nonspecific products were identified using a melt curve analysis. Fluorescence of incorporated

SYBR Green-1 dye (Molecular Probes; Eugene, OR) into newly synthesized DNA was measured. Prior to calculating the relative expression of each gene using the comparative threshold (CT) method [25], a validation study was conducted to ensure that efficiencies of the target and reference were approximately equal. Real-time PCR was performed on serial dilutions of the target and normalizer in separate tubes. Target and normalizer CT values were obtained for each serial dilution and used to calculate the difference for each dilution ( $\Delta$ CT). Efficiencies of both amplicons were approximately equal for all primers and the log input amount *versus*  $\Delta$ CT plot had a slope of approximately zero.

## 2.6 Human oligonucleotide microarray data acquisition

Agilent Human 22K microarray slides (Human 1A, v2) were scanned using a Scan Array 5000 confocal scanner (Packard BioScience; Meriden, CT) with a 10  $\mu$ M resolution and excitation/emission wavelengths of 540/570 nm for Cy3. Each chip contained 60-mer oligonucleotide probes (Agilent Technologies Canada) for 22 575 features, with  $\sim$ 18 000 well characterized, full-length human genes, and several control features. ImaGene 6.0 (Biodiscoveries; El Segundo, CA) was used to quantify the 16-bit grayscale image files. ImaGene uses an adaptive spot finding method to acquire spot intensities from mean pixel values and automatically flags poor quality spots.

## 2.7 Microarray data preprocessing and normalization

The Cy3 quantification data files for each array were read into SAS 9.1.3 (SAS Institute; Cary, NC). For each array the background noise was measured by taking the trimmed mean plus three times the trimmed SD of the median signal intensities of the (–)3xSLv1 probes. There were 162 (–)3xSLv1 probes spotted on each array. Probes that had median signal intensities less than this value were flagged to be absent; otherwise the probe was flagged to be present. The signal intensity data were then transformed in R [26] using the variance-stabilization and normalization (VSN) method. Details on the VSN are discussed in Huber *et al.* [27].

After applying the VSN transformation, the data were globally normalized using a mixed model following Wolfinger *et al.* [28]. This technique normalizes the transformed signal intensities by removing systematic biases that may be present in the data. The mixed model was applied in SAS using the PROC Mixed procedure. The model included one fixed effect, date of hybridization, and one random effect, array. The residuals from the mixed effect model were then treated as the normalized expression values. As a quality control check, a Spearman correlation analysis was conducted to assess for the similarity in response across all genes, for each individual sample within each treatment group. The results indicated a high level of correlation in all groups (Table 1), indicating a consistent response within

**Table 1.** Spearman correlation analysis of the similarity in response across all genes, for each individual sample within each treatment group

Cell line	Group	Time (h)	Spearman correlations		
			Minimum	Median	Maximum
U87MG	Positive control	24	0.844	0.881	0.908
	Negative control	24	0.878	0.906	0.938
	Sham	24	0.904	0.921	0.944
	0.1 W/kg	24	0.842	0.891	0.941
	1 W/kg	24	0.892	0.924	0.941
	10 W/kg	24	0.840	0.904	0.938
MM6	Positive control	6	0.603	0.762	0.848
	Negative control	6	0.837	0.895	0.960
	Sham	6	0.821	0.880	0.952
	1 W/kg	6	0.869	0.907	0.928
	10 W/kg	6	0.900	0.939	0.957
	Positive control	24	0.600	0.785	0.857
	Negative control	24	0.860	0.904	0.959
	Sham	24	0.866	0.877	0.922
	1 W/kg	24	0.772	0.831	0.909
	10 W/kg	24	0.825	0.857	0.920

individual treatment groups across all five independent experiments. It was observed that the correlation was slightly lower in the positive control group, presumably due to the high number of differentially expressed genes adding variance to the model.

## 2.8 Statistical analysis

For the U87MG cell line, a randomized balanced block design [29, 30] was used to analyze the data. The design was blocked using the date of hybridization. The treatment effect comprised six levels (Negative Control, Positive Control, Sham Control, and three RF field exposures at 0.1, 1.0, and 10 W/kg). Five biological replicates *per* condition were used for a total of 30 microarrays. An unbalanced block factorial design was employed for the MM6 cell line analysis. The design was blocked using the date of hybridization and the factors examined included treatment (five levels; Negative Control, Positive Control, Sham Control, and two RF field exposure levels at 1.0 and 10.0 W/kg), and time-point (two levels; 6 and 24 h). Five biological replicates *per* condition were used for a total of 50 independent microarrays. After removing three arrays due to poor data quality, the final analysis on the MM6 samples included data from 47 microarrays.

The normalized expression values were analyzed using the Microarray Analysis of Variance (MAANOVA) library in R [31]. For the U87MG cells, the analysis consisted of six contrasts of interest. For the MM6 cells, the MAANOVA model incorporated the treatment, time, and a treatment by time interaction resulting in ten contrasts of interest. A first

set of contrasts for each cell line was used to identify genes that were differentially expressed between the positive, negative, and sham controls. A second set of contrasts was then used to identify any probes that were differentially expressed between the Sham and RF field exposed samples.

The James–Stein shrinkage estimator (Fs) *F*-test was used to test for an overall treatment effect. This test is a more efficient and robust approach for detecting differentially expressed genes when there is limited information to estimate the gene specific variance components [32]. Since the Fs *F*-test is not a standard *F* statistic, the null distribution of the test statistic has to be established using permutation analysis. In this analysis the *p*-values for the Fs test was based on 10 000 permutations using residual shuffling. The Fs *F*-test *p*-values were then adjusted for the false discovery rate (FDR) [33]. Contrasts between the sham and other treatment groups in each analysis were conducted using the Fs testing procedure. Estimated marginal means also known as least square means [34, 35] were determined for each group. These means are a function of the model parameters and are adjusted for the other factors in the model such as date of hybridization. The least square means were then used to estimate the fold change for each contrast that was tested. Differentially expressed genes were those that were significant ( $p < 0.05$ ) by MAANOVA, present in at least three of 30 microarrays for U87MG cells and three of 47 microarrays for MM6 cells, and had a minimum fold change of  $\pm 1.35$ .

Semiquantitative RT-PCR data on HSP gene expression for the U87MG cell line was also analyzed in a two-step process. First, a one-factor repeated measures design ANOVA was used to compare the sham, negative, and positive control groups for each HSP. If the ANOVA was found to be significant ( $p < 0.05$ ), then the data was subjected to Duncan's multiple range test to ensure that controls were responding appropriately; namely that the sham and negative controls do not differ significantly from each other, but differ significantly from the positive (heat-shock) control. If these conditions were met, the data from the sham and the RF-exposed groups for each of the HSPs tested were then subjected to a second one factor repeated design ANOVA. Dunnett's multiple comparison test was used to compare sham controls to RF-exposed groups if significance was detected by the ANOVA ( $p < 0.05$ ).

### 3 Results

#### 3.1 Temperature monitoring

To ensure that possible biological effects observed in our study were not related to thermal confounding, the temperature in all RF-exposed samples and the sham treatment group were monitored over the course of the entire exposure period. Temperature data were collected every 60 s in the media of each of the samples. No major fluctuations in temperature were detected over the course of the exposure period as the

temperature in the samples for all experiments remained in the range of  $37.0 \pm 0.5^\circ\text{C}$  (data not shown). This was the case for both the U87MG and MM6 cell-line experiments.

#### 3.2 Hierarchical clustering of genes

Hierarchical cluster analysis was performed to examine the relationship of global gene expression patterns among treatment groups. Data obtained from both the MM6 and U87MG cell lines revealed clustering among the positive (heat-shock) control samples across separate experiments (Fig. 1). However, no apparent clustering of samples was observed among the sham or RF-exposed treatment groups, indicating that RF-treatment produced no measurable effect on global expression patterns.

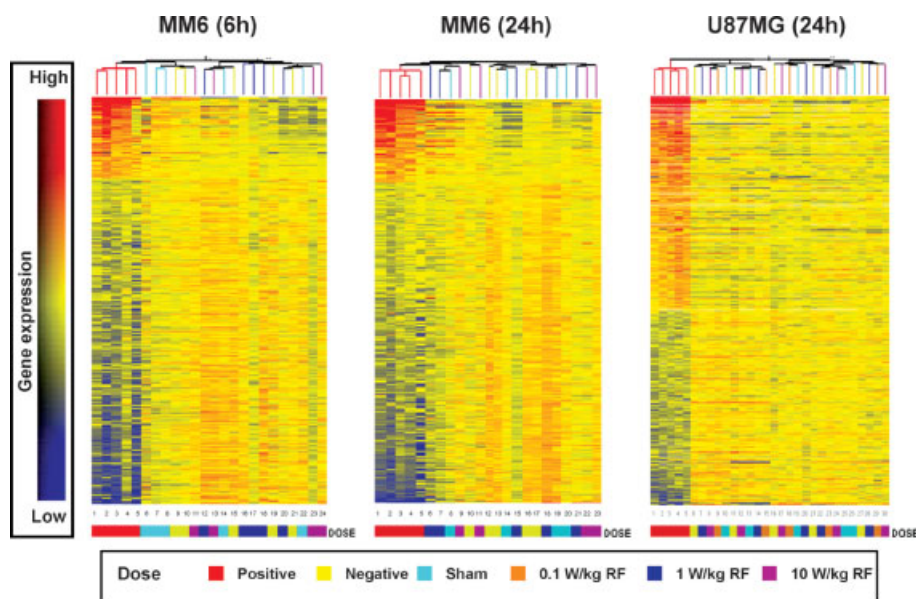
#### 3.3 Differential gene expression

To mine for differentially expressed genes, MAANOVA was employed. Significant genes identified by MAANOVA were then further filtered on flags and a minimum 1.35-fold cut-off. The results from the MAANOVA analysis showed no differentially expressed genes in the RF-exposed groups relative to the sham control in the U87MG cell line at 6 h following a 24 h RF field exposure, prior to any filtering based upon minimum fold-change. Similarly, no evidence of differential gene expression was identified in the MM6 cell line either immediately following or at 18 h following a 6 h RF field exposure. In contrast, differential gene expression was detected in the positive (heat-shock) control in both cell-lines when compared to the sham and negative control groups. A total of 439 genes were differentially expressed in the U87MG cell line exposed to heat-shock conditions. Of these genes, 236 were found to be up-regulated and 203 were down-regulated when compared to the negative- and sham-control groups. For the MM6 cell-line, a similar effect was seen in the positive control at both the  $T = 6$  and 24 h time-points. Statistical analysis revealed differential expression of a total of 2368 genes at the  $T = 6$  h time-point and 2420 genes at 18 h postexposure ( $T = 24$  h) in the positive (heat-shock) control group relative to the sham- and negative-control groups. At the  $T = 6$  h time-point, 437 genes were found to be up-regulated and 1931 were down-regulated whereas the  $T = 24$  h time-point had 453 up-regulated genes and 1967 down-regulated. Interestingly, a total of 1929 heat responsive genes in MM6 cells were observed to be differentially expressed at both the  $T = 6$  and 24 h time-points. These genes were comprised of a variety of functional classes including: stress response genes (e.g. HSPs), molecular chaperones, cell cycle control genes, transcriptional factors, and proteins of unknown function.

#### 3.4 Validation of positive control genes by RT-PCR

To confirm our microarray observations, regulation of HSPs was also investigated using semiquantitative RT-PCR in all





**Figure 1.** Hierarchical cluster analysis of gene expression patterns in two human-derived cell lines exposed to 1.9 GHz RF fields. A condition tree was applied using a standard correlation analysis in GeneSpring 7.2, for each of the following conditions: (a) MM6 cells immediately following a 6 h RF field exposure ( $T = 6$  h), (b) MM6 cells at 18 h following a 6 h RF field exposure ( $T = 24$  h), and (c) U87MG cells at 6 h following a 24 h RF field exposure. The data were normalized to the 50th percentile and the normalized intensity for each gene is represented as a block, with red and blue blocks representing high and low expression respectively, while yellow blocks are close to the 50th percentile. The samples are each shown as branches of the tree. The data show that gene expression profiles for both cell lines clustered for samples from the positive controls, indicating that the majority of variance in the data can be attributed to these variables. There was no apparent clustering for samples within the sham, negative control or RF-treated groups.

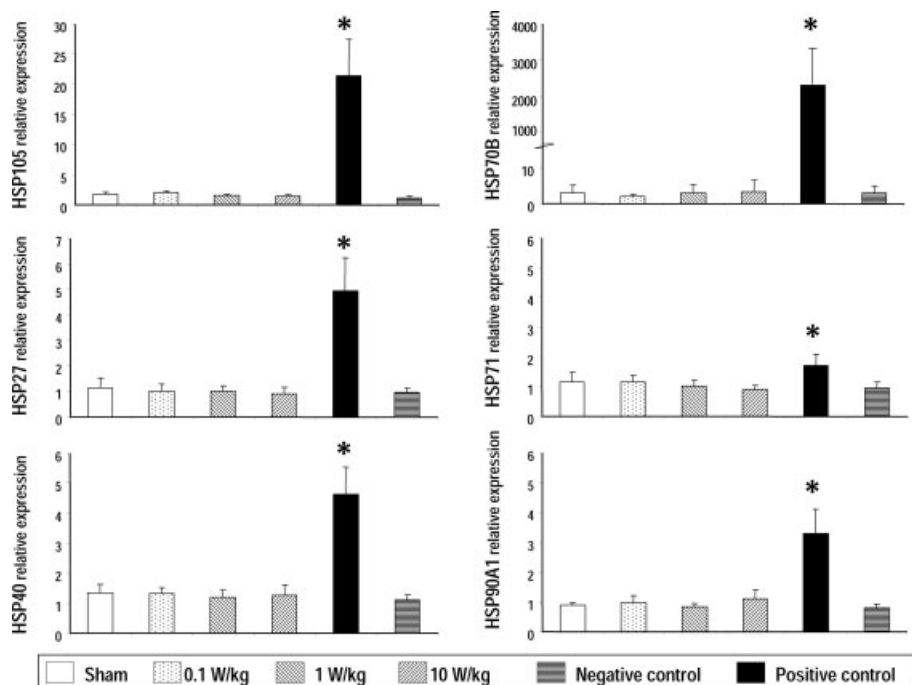
samples. No changes in HSP gene expression were observed in the negative control or the RF-exposed samples, relative to the sham control in the U87MG cells (Fig. 2). However, the positive (heat-shock) control samples showed significant up-regulation of a number of HSPs. Microarray and RT-PCR analyses obtained comparable fold-change induction for several HSPs investigated in the positive (heat-shock) control group relative to the sham exposed control for U87MG cells (Table 2). Similarly for the MM6 cell-line, there was no evidence of altered HSP expression in any of the RF exposed samples, relative to the sham- or negative control groups [15]. However, microarray analysis confirmed the up-regulation of HSP70 and HSP27 in the positive (heat-shock) control samples detected previously using RT-PCR [15], and fold-changes measured using RT-PCR and microarray were similar at both time-points (Table 2).

#### 4 Discussion

The use of DNA microarrays is becoming increasingly popular for high-throughput analysis of differential gene expression. However, a number of important flaws in study design, data acquisition, and statistical analysis have emerged in the growing numbers of studies published using this technology. First, despite the high cost of microarray slides and reagents, an experiment requires enough biologi-

cal replicates to carry out a proper statistical analysis of the data. Lack of biological replication will result in false positive and/or negative detection. Second, preprocessing steps including applying the appropriate normalization, filtering, and quality control checks are required before proceeding to data analysis [36–39]. Appropriate statistical approaches must be applied to correctly model nuisance parameters and interpret real biological variability [37]. Lastly, validation of microarray results is required using an alternative technology [40]. It is imperative to take all of these factors into account before undertaking and carrying out an experiment using DNA microarrays. In this study we designed our experiment such that the reliability of our generated dataset was not compromised as a result of poor experimental design.

We assessed the effect of short-term RF field exposure on two human-derived cell-lines using Human 1A (v2) oligonucleotide microarrays. The experimental design employed five independent experiments with 5–6 conditions *per* experiment at each time-point examined. As such, 30 independent hybridizations were performed on nonpooled RNA for U87MG cells, while 47 independent hybridizations were performed on nonpooled RNA for MM6 cells. Under these conditions, a relatively large number of independent biological replicates were included to reduce the probability of aberrant false-positive events resulting from chance and/or slight differences in culture conditions. In addition, our data



**Figure 2.** Relative HSP mRNA expression in U87MG cells. RNA extracted from U87MG cells was analyzed for gene expression using primers specific for HSP40, HSP70B, HSP71, HSP90AA1, HSP27, HSP105. Data represent the mean  $\pm$  SEM from five independent experiments. Asterisks represent significance differences ( $p < 0.05$ ) when compared from the sham treatment group.

**Table 2.** Comparison of fold changes in heat shock gene expression in the positive control samples, relative to the sham control group, determined by RT-PCR and by Agilent Human 1A (v2) microarrays in the U87MG and MM6 cell lines. Results depict the mean  $\pm$  SEM from five independent experiments

Cell line	Target gene	Gene Bank accession number	Time (h)	Fold change (Microarray)	Fold change (RT-PCR)
U87MG	HSP27	NM_001540	24	3.9	4.9 $\pm$ 1.3
	HSP40	NM_006145	24	17.3	4.6 $\pm$ 0.9
	HSP70B	NM_002155	24	54.3	2279 $\pm$ 813
	HSP71/HSPA1A	NM_005345	24	87.1	1.7 $\pm$ 0.4
	HSP90AA1/HSPCA	NM_005348	24	1.9	3.3 $\pm$ 0.8
	HSP105	NM_006644	24	5.2	21.4 $\pm$ 6.0
MM6	HSP27	NM_001540	6	6.1	10.2 $\pm$ 3.0
			24	4.9	12.1 $\pm$ 5.2
	HSP70B	NM_002155	6	97.0	5549 $\pm$ 2196
			24	74.4	8038 $\pm$ 3520

were analyzed using rigorous statistical techniques that controlled for FDR by using the James–Stein shrinkage *F*-test. This test is known to be a powerful and robust technique to test differential gene expression as it does not suffer from bias of the pooled variance approach and therefore minimizes the generation of false positive or negatives that can occur when a common variance is assumed [32]. The SAR levels chosen (0.1–10 W/kg) in the current study represent a range of approximately 1/10 to 10  $\times$  that of the peak SAR limit (1.6 W/kg) recommended by the IEEE International Committee on Electromagnetic

Safety (ICES) [41], and are relevant for the ongoing health risk assessment of RF fields.

Under the experimental conditions used in this study, we found no evidence that nonthermalizing 1.9 GHz RF field exposure affected gene expression in cultured U87MG cells at 6 h following a 24 h exposure or in MM6 cells immediately following a 6 h exposure or 18 h postexposure, relative to the sham- or negative (incubator)-control groups. The data obtained from this study support our previous results where we found no evidence of altered gene expression at 6 h following a 4 h exposure of U87MG cells to RF fields [22]. As

with our previous study, the current study also included a positive heat-shock control group in our experimental design. Gene expression changes in this treatment group revealed changes in expression of a number of genes when compared to the sham- and negative (incubator)-control groups. For the U87MG cell-line, RT-PCR confirmed an increased expression of HSP27, HSP40, HSP70, HSP90, and HSP105 in the positive (heat-shock) control group, but also the lack of response in the RF-exposed groups relative to the sham control group (Fig. 2). In the MM6 cell-line, HSP27 and HSP70 along with the proto-oncogenes *c-fos*, and *c-jun* were shown to be up-regulated in the positive control group relative to the untreated controls [16].

The results of this study support previous investigations demonstrating a lack of biological effect from nonthermal RF fields. A recent study by Hirose *et al.* [20] showed no change in p53-related genes in glioblastoma cells exposed to 2.145 GHz W-CDMA or CW signals at SARs of 80, 250, and 800 mW/kg. Whitehead *et al.* [42] investigated C3H10T1/2 mouse cells exposed to 847.74 MHz CDMA and 835.2 MHz frequency-division-multiple-access (FDMA)-modulated RF fields at 5 W/kg for 24 h. The number of genes with at least a 1.3-fold change relative to sham was less than or equal to the expected number of false positives. The authors concluded that the RF fields had no statistically significant effect on gene expression and that the genes identified are likely false positive responses. In an attempt to limit the number of false positives reported from microarray analysis, Zeng *et al.* [21] used duplicate GeneChips to form four pair-wise comparisons between RF- and sham-exposed groups. While no statistically significant differences were observed at a SAR of 2.0 W/kg, five differentially expressed genes were observed at a SAR of 3.5 W/kg. However, none of these genes could be confirmed by RT-PCR analysis. Therefore, the authors concluded that 1800 MHz RF fields at intensities up to a SAR of 3.5 W/kg for 24 h did not result in reproducible changes in gene expression in MCF-7 cells.

In contrast, a number of recent microarray studies have reported differential gene expression from RF field exposure. In a study by Lee *et al.* [18], 2.45 GHz RF field exposure was reported to cause differential expression of over 750 genes in HL60 cells after a 6 h exposure period. However, it should be noted that this study effectively performed statistical analysis on data from a single independent experiment. Furthermore, the statistical analysis techniques employed did not correct for multiple comparisons. Therefore, it is not surprising that this study reported a relatively large number of differentially expressed genes between groups. Due to the absence of true biological replicates, correction for multiple comparison testing or external validation of observed changes using alternate methodologies, little confidence can be given to these data. Similarly, Remondini *et al.* [19] reported differential gene expression in several human-derived cell lines following RF field exposure. The approach used in this investigation also suffered from critical methodological flaws. In this study, RNA was isolated from two to five inde-

pendent exposure experiments and pooled prior to generating cDNA. Thereafter, only one cDNA sample for each of the sham and RF field exposed groups were hybridized for each experimental condition. As multiple independent pools were not available in this study, data analysis was then carried out based on only a single independent hybridization *per* experimental group. In order to estimate the true biological variance for statistical inference, multiple independent pools of RNA are required. Although each gene was spotted four times on the same nylon membrane, the authors also failed to produce any meaningful technical replicates. Duplicate spots on a single microarray slide are not independent, as the same cDNA sample is hybridized onto the same membrane at the same time, and therefore replicate spots on a microarray do not constitute valid technical replicates. Multiple hybridizations from the same-pooled cDNA sample onto separate arrays would be required. As such, the statistical inference testing employed by the Remondini *et al.* [19] is based on unsubstantiated assumptions of biological and technical variance in their experimental model. As a result, gene specific variance components cannot be well estimated and outliers cannot be appropriately identified. In addition, the study by Remondini *et al.* [19] also failed to provide any external validation of their findings, thereby limiting the conclusions that can be drawn from this study.

In conclusion, we found no significant changes in gene expression in glioblastoma and monocytic cells resulting from 1.9 GHz RF field exposures. Most studies to date that have reported differential gene expression following RF field exposure do not adhere to many of the most fundamental aspects of well-designed microarray studies. As a consequence, the conclusions reached in those studies must be interpreted with caution as a result of serious shortcomings in the experimental design and statistical analysis of the microarray data. Future well designed high-throughput genomics and proteomics studies may assist in the evaluation of possible nonthermal bioeffects from RF field exposure.

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