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Characterization of biological effect of 1763 MHz radiofrequency exposure on auditory hair cells

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Abstract

Purpose: Radiofrequency (RF) exposure at the frequency of mobile phones has been reported not to induce cellular damage in *in vitro* and *in vivo* models. We chose HEI-OC1 immortalized mouse auditory hair cells to characterize the cellular response to 1763 MHz RF exposure, because auditory cells could be exposed to mobile phone frequencies.

Materials and methods: Cells were exposed to 1763 MHz RF at a 20 W/kg specific absorption rate (SAR) in a code division multiple access (CDMA) exposure chamber for 24 and 48 h to check for changes in cell cycle, DNA damage, stress response, and gene expression.

Results: Neither of cell cycle changes nor DNA damage was detected in RF-exposed cells. The expression of heat shock proteins (HSP) and the phosphorylation of mitogen-activated protein kinases (MAPK) did not change, either. We tried to identify any alteration in gene expression using microarrays. Using the Applied Biosystems 1700 full genome expression mouse microarray, we found that only 29 genes (0.09% of total genes examined) were changed by more than 1.5-fold on RF exposure.

Conclusion: From these results, we could not find any evidence of the induction of cellular responses, including cell cycle distribution, DNA damage, stress response and gene expression, after 1763 MHz RF exposure at an SAR of 20 W/kg in HEI-OC1 auditory hair cells.

Keywords: *Radiofrequency exposure, cell cycle, DNA damage, stress response, gene expression*

Introduction

Radiofrequency (RF) exposure does not transfer high enough energy to break the covalent bonds of macromolecules, but these low energy stimuli might be enough to induce molecular responses like cell proliferation or cell death (Moulder et al. 1999). However, there have been many epidemiological reports on the relationship between RF exposure and human cancers. For example, the brain is the most important target tissue to study the biological effect of RF exposure in mobile phone users (Hardell et al. 1999).

There was a report on the increase in tumors ipsilateral to the side of the head on which subjects recalled phone use (Hardell et al. 2002), but this was not substantiated by other studies (Muscat et al. 2000). RF exposure to rat heads did not affect the incidence, malignancy, volume, multiplicity, latency, or fatality associated with any kind of neurogenic tumor (Zook and Simmens 2006).

Alteration of the cognitive and physiological function of brains after exposure to mobile phone frequency RF has been reported by several electrophysiological studies (Curcio et al. 2005). In this study, RF exposure induced measurable changes in human brain electrical activity, particularly in the alpha frequency band (8–13 Hz) over the posterior regions of the scalp. In addition, rats that were exposed to RF showed neuronal damage in the

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cortex, hippocampus, and basal ganglia (Salford et al. 2003). However, this work was not reproduced by others (Joubert et al. 2007), and there are a number of points to consider regarding whether RF exposure can affect the human brain and its subsequent output in the form of cognition and behavior.

If RF exposure from mobile phone use can affect hearing function, we need to check the sensory elements of the auditory system. In the inner ear, the organ of Corti contains sensory elements, such as a single row of inner hair cells (IHC), a triple row of outer hair cells (OHC), and different types of supporting cells. Hair cells are the sensory neurons for auditory and vestibular functions in the inner ear. Once the auditory hair cells are damaged, hearing and vestibular impairment can occur. In this sense, it would be very important to know whether any biological alteration in auditory hair cells can occur by mobile phone frequency exposure.

Gene expression profiling with microarrays can provide valuable information on the characteristics of certain physiological and pathological conditions. For example, the gene expression profiles of γ-irradiated Jurkat cells showed p53-independent activation of the nuclear factor-κB (NF-κB) pathway (Park et al. 2002). HL-60 cells that were exposed to 2.45 GHz RF were examined for their expression patterns to select for genes that were related to RF exposure (Lee et al. 2005). Gene expression and cell cycle distribution also were measured in monocytic U937 and neuroblastoma SK-N-SH cells after Global System of Mobile communications (GSM) exposure, which resulted in no significant differences between sham-exposed and RF-exposed cells (Gurisik et al. 2006).

In the present study, we used HEI-OC1 mouse auditory cells that were derived from long-term cultures of 'Immortomouse' cochleas (Kalinec et al. 1999). These cells express several molecular markers that are characteristic of organ of Corti sensory cells. To examine the biological effect of 1763 MHz RF exposure from mobile phone use, we checked several parameters, such as changes in cell cycle, DNA damage, and the stress response, in RF-exposed HEI-OC1 cells. We also compared the gene expression patterns of RF-exposed cells against sham-exposed cells using a microarray to monitor any alteration in transcription levels.

Material and methods

Antibodies and reagents

Antibodies against beta-actin and heat shock proteins (HSP), such as Hsp27, Hsp70, and Hsp90, and horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), p38, phospho-JNK (Thr183/Tyr185), phospho-ERK (Thr202/Tyr204), and phospho-p38 (Thr180/Tyr182) were purchased from Cell Signaling (Beverly, MA, USA). The molecular weight standards for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Bicinchoninic acid (BCA) Protein Assay Reagents were obtained from PIERCE (Rockford, IL, USA). The enhanced chemiluminescence (ECL) detection kit was purchased from Amersham Biosciences (Little Chalfont, UK).

Cell culture and RF exposure

Conditionally immortalized HEI-OC1 mouse auditory cells were kindly provided by Dr Kalinec (Kalinec et al. 2003). Cells were cultured under permissive conditions (33°C, 10% CO2) in high-glucose Dulbecco's Modified Eagle's medium (DMEM; GIBCO/BRL, Gaithersburg, MD, USA) containing 10% heat-inactivated fetal bovine serum (FBS, GIBCO/BRL, Gaithersburg, MD, USA) and 50 U/ml gammainterferon (Genzyme, Cambridge, MA, USA).

The exposure system that was specifically designed for this study was reported previously (Lee et al. 2006a). For the personal communications service (PCS) exposure system, a real code division multiple access (CDMA) signal at 1762.5 MHz was applied. Briefly, cells were exposed to 1763 MHz RF in 100 mm Petri dishes containing 18 ml of medium, and sham exposures were carried out in parallel with the same kind of chamber with no antenna. After a 24-h or 48-h exposure at a specific absorption rate (SAR) of 20 W/Kg, the cells were harvested at the indicated time. During exposure, the temperature in the chamber was maintained at 33 ± 0.2°C by circulating water within the cavity. We repeated sets of experiment three times to collect biological triplicates for each sample.

Cell cycle analysis

To observe cell cycle phases, cells were harvested, fixed with 70% alcohol at 4°C, and then stained with 50 ug/ml propidium iodide (PI). The stained cells were analyzed for DNA content by flow cytometry using a FACS Calibur (Becton Dickinson, Franklin Lakes, NJ, USA).

Comet assay

To check DNA damage, we performed the comet assay as described previously (Kim et al. 2006).

Briefly, 1% normal melting point agarose (NMA) (Ameresco, Solon, OH, USA) and 0.5% and 1% low melting point agarose (LMA) (Ameresco, Solon, OH, USA) in phosphate-buffered saline (PBS) were prepared to precoat the slides. Onto the slides, we applied a mixture composed of an equal volume of cells and 1% LMA, and we submersed the slides in lysis solution (2.5 M NaCl, 100 mM ethylenedinitrilotetraacetic acid (EDTA), 10 mM Tris–HCl, pH 10; 1% Triton X-100 and 10% dimethyl sulfoxide(DMSO), pH 10).

Next, the slides were placed into unwinding buffer (1 mM EDTA and 300 mM NaOH, pH 13) for 20 min and separated by electrophoresis at 25 V and 300 mA (0.8 V/cm) for 20 min. After electrophoresis, the slides were neutralized by neutralization buffer (400 mM Tris-HCl, pH 7.4) and stained with 10 μg/ml ethidium bromide (EtBr). The stained slides were examined using a Komet 4.0 image analysis system (Kinetic Imaging, Liverpool, UK) that was fitted with an Olympus BX50 fluorescence microscope equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. For each treatment group, two slides were prepared, and 50 randomly chosen cells (total 100 cells) were scored manually for each slide. The parameter, Olive tail moment (= (Tail-mean − Head-mean) * Tail% DNA/100), was calculated automatically using the Komet 4.0 image analysis system, which was used for global comet description.

Western blotting

Cellular extracts were prepared by treating cells with extraction buffer (1% Triton X-100, 0.5% sodium deoxycholate, 20 mM Tris-HCl, pH 7.5, 12 mM b-glycerophosphate, 150 mM NaCl, 5 mM ethylene glycol tetraacetic acid (EGTA), 10 mM NaF, 3 mM dithiothreitol (DTT), 1 mM sodium orthovanadate, 1 mM phenymethysulfonyl fluoride, 20 mg/ml aprotinin), and the protein concentrations were measured with BCA Protein Assay Reagents. Equal amounts of total proteins were loaded and separated in 10% SDS-PAGE gels. After transfer of proteins to nitrocellulose membranes, the membranes were blocked and incubated with the indicated antibodies for 4 h at room temperature. The immune complexes were detected by horseradish peroxidase-conjugated secondary antibody and visualized using an ECL detection kit. Signal intensities were quantified by scanning densitometry. The signal was scanned with EPSON Perfection 1660 Photo, and the signal intensity was determined with the ImageJ Program (http://rsb.info.nih.gov/ij/). The figures show the level of density as a ratio after normalization with the internal control density.

Microarray and data analysis

Samples in each group were harvested in triplicate, and total RNA was extracted by dissolving cells in TriZol and purified using Qiagen RNAeasy columns (Park et al. 2002). We used the Applied Biosystems 1700 full genome expression mouse microarray, which included 32,000 mouse genes from the public and Celera databases (http://www.pantherdb.org/). The figures show the level of density as a ratio after normalization with the internal control density.

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Results

Cell cycle phase does not alter on RF exposure

To investigate the possible effect of RF exposure on the cell cycle, we exposed log-phase HEI-OC1 mouse auditory cells to 1763 MHz RF at an SAR of 20 W/kg for 24 h (Figure 1A) and 48 h (Figure 1B). According to their DNA content, we separated cells into four phases, sub-G0/G1, G1, S, and G2/M, using a fluorescence-activated cell sorter (FACS) analyzer. The pattern of the cell cycle phases of RF-exposed cells was not different from that of sham-exposed cells. In both groups, the proportion of sub-G0/G1 phase cells was extremely low. The proportion of cells in G1 phase in the 48-hr exposure group was higher than in the 24-h exposure group, but there was no significant difference between RF- and sham-exposed cells. On the other hand, the proportion of cells in S and G2/M phases was decreased in the 48-h exposure group, which might be due to the increase in G1 phase cells. The number of S phase cells seemed to be higher in the 48-h RF-exposed cells (Figure 1B), but it was not statistically significant. The overall patterns of the cell cycle phases in sham- and RF-exposed cells did not show any significant change.

RF exposure does not induce DNA damage

We checked DNA damage using the comet assay in HEI-OC1 auditory cells after 1763 MHz RF exposure at an SAR of 20 W/kg for 6, 24, and 48 h. Two comet assay parameters, Olive tail moment and tail length, were measured to examine DNA damage on RF exposure (Table I). Both parameters were unchanged in the RF-exposed group, even after 48 h of exposure, in comparison with the sham group. Olive tail moment in the 1763 MHz-exposed group (1.99 ± 0.05) was higher than in the sham-exposed group (1.26 ± 0.22) after 24 h, but the Wilcoxon's rank test revealed that there was no significant difference between the sham- and RF-exposed groups. From this result, we could not find evidence of DNA damage using the comet assay after 1763 MHz RF exposure at a 20 W/kg SAR for 48 h.

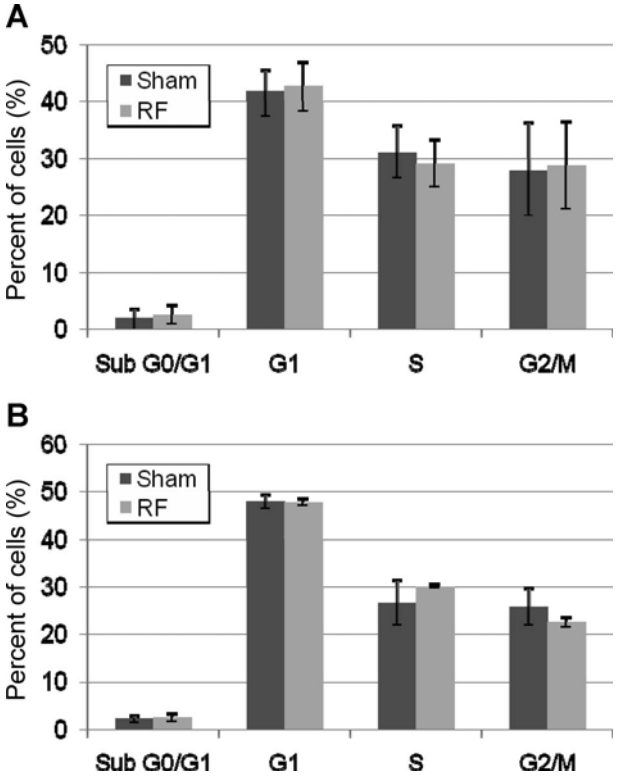

Figure 1. The biological effects of 1763 MHz RF on the cell cycle. HEI-OC1 cells after exposure to 1763 MHz at an SAR of 20 W/Kg for 24 h (A) or 48 h (B) were analyzed their distribution of DNA content using FACS.

Table I. Comet assay of HEI-OC1 cells exposed to 20 W/kg SAR of 1,763 MHz RF.

Exposure time	Exposed groups	Olive tail moment (Mean ± SD)	Tail length (Mean ± SD)
6 h	Sham	1.27 ± 0.43	19.63 ± 3.50
	RF	1.30 ± 0.17	21.31 ± 2.35
24 h	Sham	1.26 ± 0.22	20.38 ± 2.19
	RF	1.99 ± 0.05	21.25 ± 1.34
48 h	Sham	1.08 ± 0.49	20.93 ± 3.15
	RF	1.22 ± 0.34	20.92 ± 1.83

Each data point represents the mean ± the standard deviation (SD). Representative data was from three independent experiments. Wilcoxon signed rank test showed $p > 0.05$.

Stress responses were not detected after RF exposure

Because RF exposure can be considered to be an environmental stressor, stress biomarkers such as heat shock proteins (HSP) and mitogen-activated protein kinases (MAPK) can be monitored. HEI-OC1 auditory cells were exposed to 1763 MHz RF at an SAR of 20 W/kg for 6, 12, and 24 h, and the expression levels of Hsp90, HspP70, and Hsp27 were examined by Western blot (Figure 2A). The protein levels of all HSP were unchanged by quantitative analysis of triplicate experiments (Figure 2B). The phosphorylation of MAPK such as Erk1/2, Jnk1/2, and p38 also were determined by Western blot after RF exposure at 20 W/Kg for 15, 30, 60, and 120 min. Phosphorylation of Jnk1/2 and p38 was not detected in any of the samples that were examined, while we could have signals in the positive control, heat-treated Jurkat cells ($43 \pm 0.2^{\circ}$C, 30 min) (Figure 3A). Erk1/2 was phosphorylated in both the sham- and RF-exposed cells, and the quantities of phospho-Erk1/2 signal were not changed by RF exposure (Figure 3B). We could not detect any alterations in the levels of stress proteins or in the activation of MAPKs by RF exposure in comparison with the sham exposure.

Minimal changes in gene expression patterns were detected

To detect any possible response of HEI-OC1 cells to RF exposure, we used a microarray to monitor genome-wide gene expression in sham- and RF-exposed cells. Cells were exposed once to 20 W/kg RF for 24 h and harvested cells after 5 h (PR5), and in parallel, we also harvested sham-exposed cells as a control group (SR5). After a rigorous filtering process of the microarray expression data of 32,000 mouse genes, we selected differentially expressed genes (DEG) on RF exposure using cutoffs, such as changes that were > 1.5-fold and statistically significant (false discovery rate (FDR) < 0.05) using triplicates of 15,040 genes. Only 29 genes fit these standards, including expressed sequence tags (EST) (Table II).

Fourteen genes, including 5 ESTs, were decreased by more than 1.5-fold on RF exposure. Among them, RAN binding protein 2 (Ranbp2) and serum amyloid A 2 (Saa2) were downregulated by 8.1- and

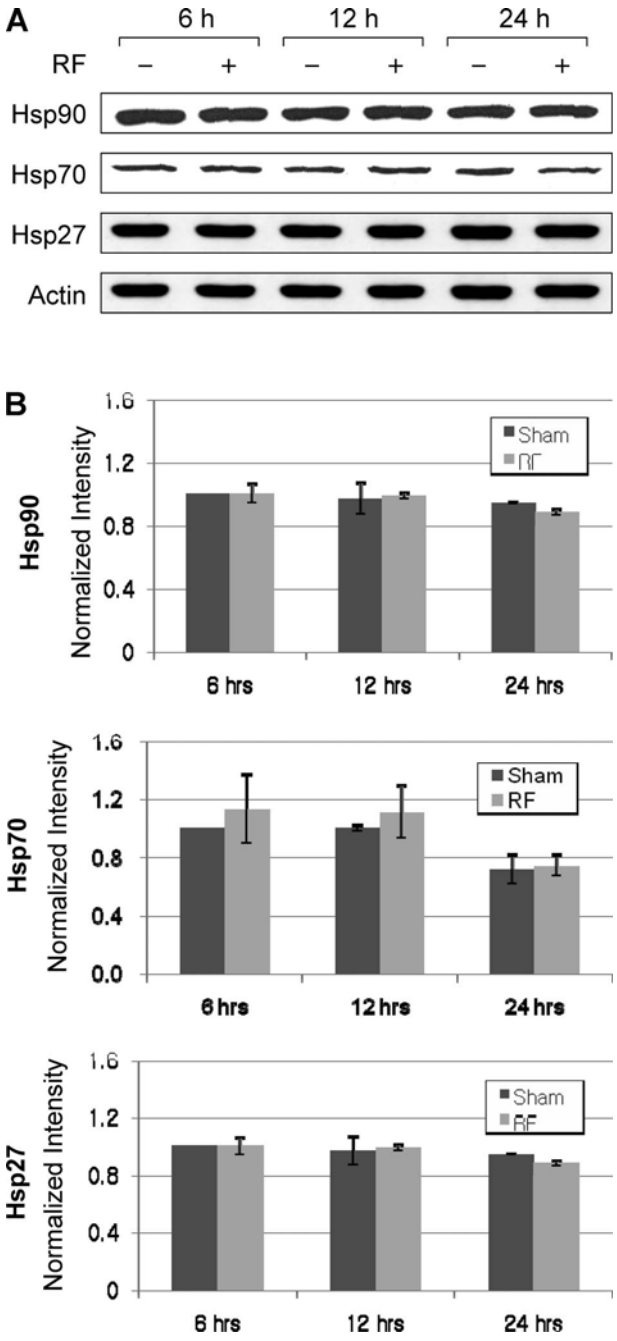

Figure 2. Effects of RF exposure on HSP levels in HEI-OC1 auditory cells. (A) Western blot analysis of HSP expression in HEI-OC1 auditory cells exposed to RF at 20 W/kg for 6, 12, or 24 h continually. After exposure, cells were harvested immediately and stored at -70°C for Western blotting. Representative data from three independent experiments are shown. (B) The intensity of each band in the Western blot analysis was measured from three independent experiments.

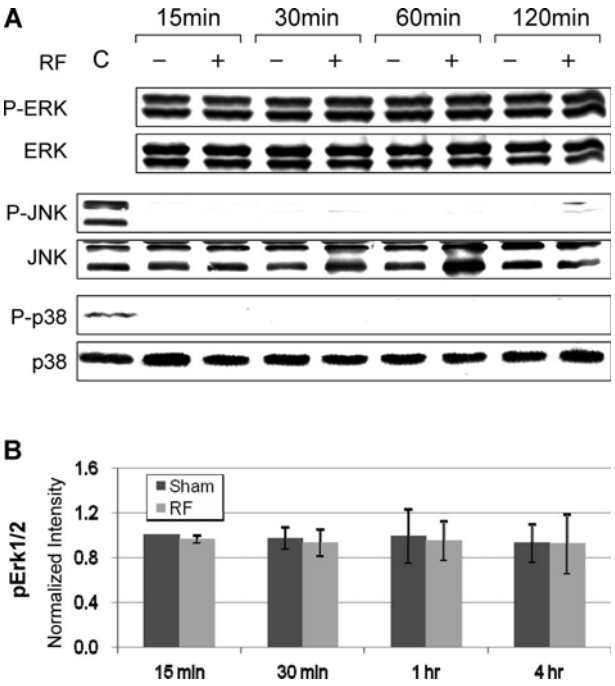

Figure 3. Effects of RF exposure on MAPK phosphorylation in HEI-OC1 auditory cells. Western blot analysis of phosphorylated MAPK in lysates prepared immediately after exposure to RF for 15, 30, 60, and 120 min. Representative data from three independent experiments are shown. A positive control (PC) was prepared from heat-treated Jurkat cells ($43 \pm 0.2^{\circ}$C, 30 min) in the detection of JNK and p38 phosphorylation. (B) The intensity of each band in the Western blot analysis was measured from three independent experiments.

2.4-fold, respectively, while the other 12 genes changed less than 2-fold. Fifteen genes were decreased by more than 1.5-fold. Nine genes, such as Isoleucine-Glutamine (IQ) motif-containing E (Iqce), olfactory receptor 438 (Olfr438), Werner helicase-interacting protein 1 (Wrnip1), leucine rich repeat-containing 25 (Lrrc25), glucosamine (Gne), gametogenetin binding protein 1 (Ggnbp1), and three ESTs, were changed by more than 2-fold. In the meantime, the 29 genes that changed on RF exposure represented just 0.09% of the total genes examined in the microarray.

Discussion

Because the ear and temporal region of the brain are the closest parts of the body that are exposed to RF from mobile phones, it is important for us to know the biological consequences in those tissues or cells. Auditory hair cells are important for hearing and vestibular function in the ear. In the European project 'GUARD', Parazzini et al. monitored the auditory function of young adults after exposure to GSM mobile phone signals, but there were no effects on hearing function (Parazzini et al. 2007). In an evaluation of the effects of cellular phones on the function of rat cochlea, no significant variation due to exposure to 900 MHz RF has been found (Galloni et al. 2005). In our experiments, we used HEI-OC1 cells under nonpermissive conditions for RF exposure. In this study, we tried to find alterations in cell

Table II. Lists of differentially expressed genes upon RF exposure in HEI-OC1 cells.

Symbol	Gene name	Log2(fold activation)	*p* value
1. Down-regulated genes			
Ranbp2	RAN binding protein 2	−3.024	0.009
Saa2	serum amyloid A 2	−1.257	0.025
Gpr39	G protein-coupled receptor 39	−0.972	<0.001
Kif14	kinesin family member 14	−0.872	0.001
Polh	polymerase (DNA directed), eta (RAD 30 related)	−0.829	0.026
Efna5	ephrin A5	−0.763	0.002
Cbara1	calcium binding atopy-related autoantigen 1	−0.729	0.032
Klk12	kallikrein 12	−0.660	0.002
Trim62	tripartite motif-containing 62	−0.655	0.032
LOC434317	EST	−0.828	0.019
9930013L23Rik	EST	−0.730	0.006
E330001B16Rik	EST	−0.712	0.020
9530062K07Rik	EST	−0.627	<0.001
LOC627737| LOC632026	EST	−0.611	0.005
2. Up-regulated genes			
Prm3	protamine 3	0.653	<0.001
Actn3	actinin alpha 3	0.709	<0.001
Col11a2	procollagen, type XI, alpha 2	0.928	0.006
Iqce	IQ motif containing E	1.030	0.036
Olfr438	olfactory receptor 438	1.296	0.005
Wrnip1	Werner helicase interacting protein 1	1.365	0.002
Lrrc25	leucine rich repeat containing 25	1.504	0.001
Gne	Glucosamine	1.765	0.005
Ggnbp1	gametogenetin binding protein 1	1.807	0.038
1700047E10Rik	EST	0.603	0.034
BB001228	EST	0.892	<0.001
4930570N18Rik	EST	0.990	0.048
AW112010	EST	1.035	0.001
LOC638580| LOC384925	EST	1.399	0.027
3110056O03Rik	EST	1.650	0.009

cycle, DNA damage, and stress responses in these cells after exposure to 1763 MHz RF for up to 48 h. Especially, we used the maximum power on our device to get a 20 W/kg SAR to know any toxic effects on the cells under this condition, but we could not find any change, even at a high SAR. In addition, we could hardly detect any significant change in the levels of whole transcripts after RF exposure for 24 h.

In previous experiments using C3H 10T1/2 mouse fibroblasts and U87MG human glioblastoma cells, exposure to 847.74 MHz RF at an SAR of 0.6 W/kg did not affect cell cycle progression (Higashikubo et al. 1999). Another experiment that exposed Mono Mac 6 human acute monocytic leukemia cells to 1800 MHz RF for 72 h also did not show any evidence of cell cycle alterations (Lantow et al. 2006). In epidemiological studies, the correlation of the pathogenesis of acoustic neuroma with mobile phone use has been proposed (Hardell et al. 1999). We used HEI-OC1 mouse auditory hair cells to check the effect on the proliferation of neurons in the ear, but we could not find any alterations in cell cycle distribution, even after 48 h of exposure. Because we kept the cells under non-permissive conditions for RF exposure, the cells could not progress in the cell cycle in our exposure schedules. When we exposed cells with RF for 48 h, the proportion of G1 phase cells was increased, but the overall distribution of cell cycle phases was unchanged. In addition, the proportion of sub-G0/G1 cells was not increased on RF, which suggests that no induction of apoptosis by RF exposure takes place either.

Vijayalaxmi and Obe reviewed that 58% of published results on RF exposure did not indicate increased damage to genetic material, as assessed by DNA strand breaks, incidence of chromosomal aberrations, micronuclei, and sister chromatid exchanges (Vijayalaxmi and Obe 2004). While 23% of investigations reported an increase in such damage in cells exposed to RF, the other 19% was inconclusive. Still, there is controversy over the biological effect of RF exposure on DNA damage, but our exposure conditions – 1763 MHz RF exposure at an SAR of 20 W/kg for up to 48 h – could not induce any measurable DNA damage in HEI-OC1 auditory cells.

While the mechanistic analysis of the physiological and pathological changes can be explained in terms of genome-wide expression analysis, the poorly defined biology of responses to unidentified toxic materials can be interpreted by patterns of gene expression. In our study, only 29 genes (18 annotated genes and 11 ESTs) were changed by RF exposure. The number is small enough to cover less than 0.1% of the whole genome examined, and there was no consistent group of functional categories, such as cell cycle and apoptosis. Especially, the number of genes that changed can be detected even in false positives. In this sense, we propose that the genes listed in Table 2 might not represent the unknown characteristics of cellular responses to RF exposure. In further studies, we need to verify the alteration of these genes and perform a functional analysis of candidate genes to understand the biological responses to RF exposure.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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