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Whole-Genome Expression Analysis in Primary Human Keratinocyte Cell Cultures Exposed to 60 GHz Radiation

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The main purpose of this study is to investigate potential responses of skin cells to millimeter wave (MMW) radiation increasingly used in the wireless technologies. Primary human skin cells were exposed for 1, 6, or 24 h to 60.4 GHz with an average incident power density of 1.8 mW/cm^2 and an average specific absorption rate of 42.4 W/kg. A large-scale analysis was performed to determine whether these exposures could affect the gene expression. Gene expression microarrays containing over 41,000 unique human transcript probe sets were used, and data obtained for sham and exposed cells were compared. No significant difference in gene expression was observed when gene expression values were subjected to a stringent statistical analysis such as the Benjamini–Hochberg procedure. However, when a *t*-test was employed to analyze microarray data, 130 transcripts were found to be potentially modulated after exposure. To further quantitatively analyze these preselected transcripts, real-time PCR was performed on 24 genes with the best combination of high fold change and low *P*-value. Five of them, namely CRIP2, PLXND1, PTX3, SERPINF1, and TRPV2, were confirmed as differentially expressed after 6 h of exposure. To the best of our knowledge, this is the first large-scale study reporting on potential gene expression modification associated with MMW radiation used in wireless communication applications. Bioelectromagnetics 33:147–158, 2012.

Key words: millimeter waves; 60 GHz; biological effects; DNA microarray

INTRODUCTION

During the last decade, the wireless applications with the most significant growth rates have been the cellular mobile networks (e.g., Global System for Mobile Communications (GSM) and Universal Mobile Telecommunications System (UMTS)) and wireless local area networks (e.g., Wireless Fidelity (WiFi) and Worldwide Interoperability for Microwave Access (WiMAX)). Due to the saturation of the lower part of the microwave spectrum and the increasing need for high data transfer rates, the operating frequencies of emerging wireless communication systems have recently shifted toward the millimeter wave (MMW) band (30–300 GHz). In this context, applications in the 57–64 GHz band have attracted growing attention particularly for broadband indoor short-range wireless communications (e.g., Wireless High-Definition (WiHD) and Wireless Gigabit Alliance (WiGig)), and recently for body-centric

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communications within future Body Area Networks (BAN) [Zhadobov et al., 2011].

The increasing number of MMW exposure sources requires the assessment of potential biological and health effects of these radiations. Nowadays, possible health risks due to utilization of wireless technologies represent a major public concern. Therefore, it is important to evaluate potential biological impacts induced by 60 GHz radiation. In order to generate data regarding the safety of emerging wireless communications, we performed a whole-genome expression analysis to investigate potential biological effects of MMW radiations corresponding to near-future application scenarios.

At MMW frequencies, heating is the major effect related to the absorption of the electromagnetic energy by tissues, cells, and water solutions. However, wireless MMW communication systems operate at power density (PD) levels low enough (<1 mW/cm^2 for the general public) to not induce any significant thermal effect. For such incident PD levels, skin heating is typically below 0.1 °C [Zhadobov et al., 2011]. The existence of direct or combined biological effects, not directly related to the temperature rise, is still controversial.

MMW at intermediate PD (5–15 mW/cm^2) have been used in Eastern European countries for therapeutic purposes [Pakhomov et al., 1998]. In addition, several publications have shown that low- and mid-power MMW radiation may interfere with some cellular processes. For instance, it was demonstrated that MMW are able to induce anti-inflammatory effects or modify the immune status of an organism [Logani et al., 2002; Makar et al., 2003; Gapeyev et al., 2008]. Furthermore, it was shown that MMW treatment may have an analgesic effect in both human and animal models [Radzievsky et al., 1999; Rojavin et al., 2000]. This hypoalgesia can result from a direct effect of MMW on nerve cells [Radzievsky et al., 2008; Alekseev et al., 2010].

It was also observed that low-power MMW can modify the proliferation [Beneduci et al., 2005] and energetic metabolism of exposed cells [Beneduci et al., 2007]. However, these results have not been reproduced, and other studies have showed that neither the cell doubling time nor the cell cycle is significantly modified by MMW exposure [Beneduci, 2009; Zhadobov et al., 2009]. At the molecular level, 60 GHz radiation below 1 mW/cm^2 can induce structural modifications in artificial biomembranes [Zhadobov et al., 2006]. Similarly, it was shown that MMW at 53 GHz can induce physical changes of phospholipid vesicles [Ramundo-Orlando et al., 2009]. Taking into account the non-ionizing nature of MMW, it was not surprising that these radiations were not genotoxic [Vijayalaxmi et al., 2004]. On the other hand, the possibility of a proteotoxic effect has been raised. Our previous in vitro studies between 59 and 61 GHz have demonstrated that MMW have no proteotoxic effect for incident power densities close to those expected from commercial wireless communication systems (0.1–1 mW/cm^2) [Zhadobov et al., 2007; Nicolas Nicolaz et al., 2009a,b].

Among the scientific literature, very few publications focused on potential MMW effects on gene expression. Millenbaugh et al. [2008] have shown that expression of genes involved in transcriptional regulation, protein folding, oxidative stress, immune response, and tissue matrix turnover can be modified after high-power exposure (75 mW/cm^2) of rats to 35 GHz. However, as the PD was high, the results were at least partly mediated by the temperature rise. The skin injury led to subsequent recovery processes involving inflammation and tissue repair. Potential target genes of MMW radiation still remain to be identified. Since MMW are essentially absorbed by the superficial layers of skin [Zhadobov et al., 2008], the main purpose of this study was to perform a large-scale genomic analysis of primary human keratinocytes after 1, 6, or 24 h exposure to MMW at 60.4 GHz.

MATERIALS AND METHODS

Cell Culture and Exposure Protocol

Primary keratinocytes were composed of pooled cells from three patients and isolated from human neonatal foreskin discarded during circumcision. Keratinocytes were cultured at 37 °C in a humidified 5% CO_2 atmosphere. Cells were grown in conditions recommended by the supplier, on tissue culture plates coated with Collagen IV (Becton Dickinson, Franklin Lakes, NJ) in presence of defined keratinocyte serum-free media (Gibco, Carlsbad, CA). To exclude any problem of senescence or drift of the cellular population, the experiments on primary keratinocytes were randomly conducted at passages 4–7.

Three exposure durations were considered: a short-term 1 h exposure, a mid-term 6 h exposure, and a long-term 24 h exposure. One day prior to exposure, cells were seeded in a six-well culture plate at a density of 16,000 cells/cm^2, and the medium was changed just before exposure. For each experiment, cells remained in the incubator for 24 h with the generator switched on for 0 h (sham), 1, 6, or 24 h. Cells were harvested immediately after. Four

independent biological replicates per condition were used.

Exposure Set-Up and Dosimetry

The cells were exposed or sham-exposed to 60.4 GHz using the exposure set-up schematically represented in Figure 1A. The six-well tissue culture plate (8.5 cm × 12.7 cm) containing the cells was placed in the MEMMERT UE400 incubator (Fisher Scientific, Illkirch, France) at 37 °C and irradiated from the bottom by a pyramidal horn antenna. The main units and characteristics of this exposure set-up were previously described in detail [Zhadobov et al., 2009]. Sham exposures were performed under the same experimental conditions but with the generator switched off.

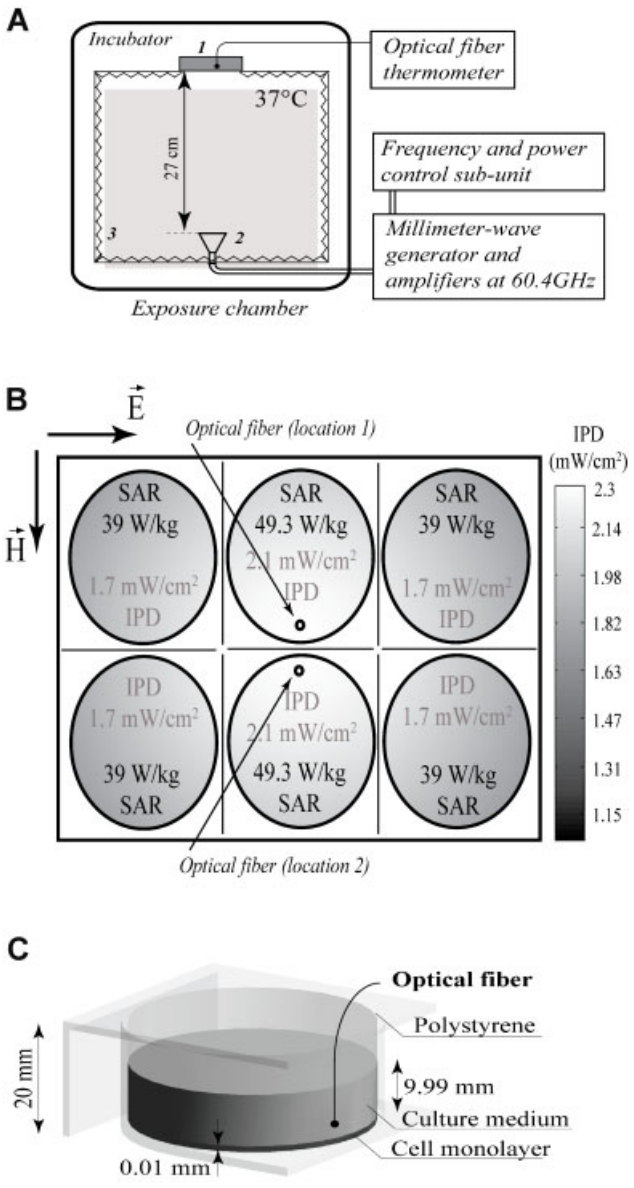

Fig. 1. **A**: Schematic representation of the exposure system: (1) tissue culture plate; (2) 17 dB gain pyramidal horn antenna (aperture dimensions 22.2 mm × 16.7 mm); (3) MMW absorbers. **B**: Incident power density (PD) distribution, average incident PD and SAR for each well, and locations of the optical fibers used for the temperature measurements. **C**: Dimensions of one well of the tissue culture plate and location of the optical fiber probe within the well.

The tissue culture plate was placed in the far-field zone of the antenna. This ensures a relatively uniform distribution of the PD over cells located in different wells. The incident PD and average specific absorption rate (SAR) within the cell monolayers have been computed numerically using the methodology described by Zhadobov et al. [2009]. Our simulation results are shown in Figure 1B. These data are given for an antenna output power of 425 mW. The peak incident PD and average PD over six wells is equal to 2.3 and 1.8 mW/cm^2, respectively. These PD values are representative of general public (1 mW/cm^2) and occupational (5 mW/cm^2) safety MMW exposure limits for far-field exposures [International Commission on Non-Ionizing Radiation Protection (ICNIRP), 1998]. The radiated power and operating frequency were carefully checked and adjusted before each exposure.

To estimate the maximal temperature elevation within the exposed samples, the temperature rise within the culture medium was locally monitored within two central wells (Fig. 1B) using a Reflex multi-channel fiber optic thermometer (Optoprim, Paris, France) with a precision of ±0.05 °C and accuracy of ±0.5 °C. The temperature dynamics were measured at the very tip of the optical fiber (diameter of 0.4 mm; Fig. 1C) with a 1 Hz sampling rate. The actual temperature increment for the exposed cells is expected to be equal to or below measured values, depending on the exact location of the cells within the well. It is worthwhile to note that the temperature rise distribution at the cell level is more uniform compared to the SAR distribution because of the thermal conductivity and convection.

RNA Isolation and Complementary RNA Labeling

RNAs from three wells were pooled together, constituting one RNA sample. Two independent RNA purifications were carried out immediately after each exposure. The first RNA preparation was used for micro-array analysis and the second one for reverse transcription-polymerase chain reaction (RT-PCR) validation (Fig. 2). Cells were washed with phosphate-buffered saline (PBS) and then harvested in lysis buffer. Total RNAs were isolated using Qiagen RNeasy columns (Qiagen, Hilden, Germany)

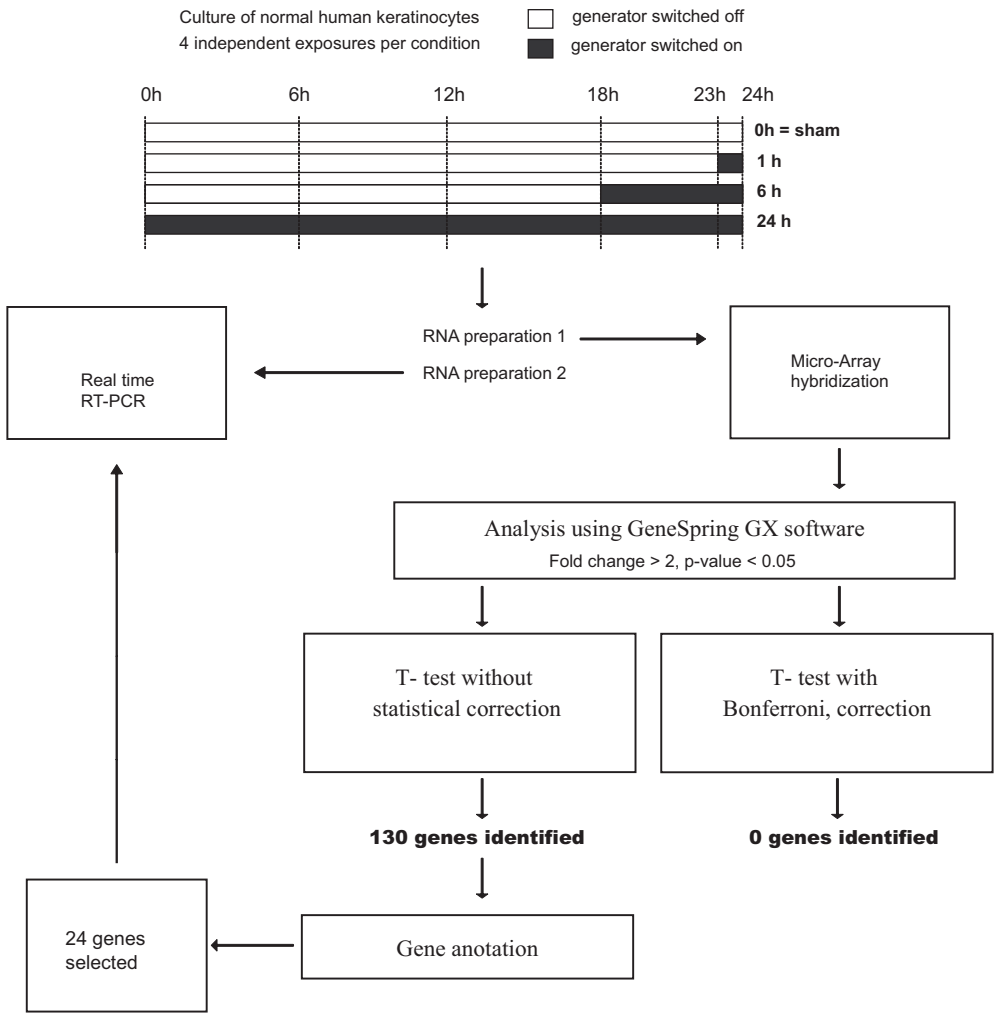

Fig. 2. Experimental protocol.

according to the manufacturer's instructions. Purified RNAs were then quantified using a Nanodrop 1000 spectrophotometer (Nanodrop Technology, Cambridge, UK), and RNA integrity was assessed using a 2100 Bioanalyzer (Agilent, Palo Alto, CA). All RNA samples had an RNA integrity number (RIN) greater than 9. RNA samples (350 ng) and RNA controls from the One-Color RNA Spike-In Kit (Agilent) were first reverse transcribed according to the manufacturer's recommendations. The resulting cDNAs were subsequently used for in vitro transcription and labeled with cyanine-3-labeled cytosine triphosphate using the Low RNA Input Linear Amplification Kit PLUS, One-Color (Agilent) according to the manufacturer's protocol. Cy3-labeled cRNAs were then purified using the RNeasy Mini Kit (Qiagen) and checked for quality with the Nanodrop 1000 spectrophotometer.

Data Analysis and Statistical Methodology

Briefly, 1.65 μg of Cy3-RNAs were hybridized to DNA microarrays using the Whole Human Genome Kit, 4 × 44 K (Agilent) according to the manufacturer's instructions. The fluorescence intensity was assessed using a DNA microarray scanner (G2565BA; Agilent), and Feature Extraction Software version 7.5 (Agilent) was used to extract signals from local background. Only features with good signal-to-noise ratios were used for further analysis. Data were log2-transformed and normalized (quantile normalization and baseline transformation) using GeneSpring GX software (Agilent). Analysis of identical replicate samples shows a slight variability, suggesting good technical quality. Moreover, the accuracy of the relative quantification was validated using the One-Color RNA Spike-In Kit. Genes were

considered significantly differentially expressed if the absolute fold change (FC; exposed vs. sham) was greater than 2 and the *P*-value was <0.05 with the Benjamini and Hochberg (B–H) procedure (multitest, R package, GeneSpring GX). A second microarray analysis was performed using a less stringent condition by removing the B–H correction and using a single *t*-test. In absence of statistical correction, some false positives might be expected and the genes identified cannot be considered as significantly differentially expressed, but as potentially affected by MMW exposure. We estimated the number of false positives by using the permutation method based on a sham versus sham comparison [Whitehead et al., 2006b]. We found around 80 potential false positives with a *t*-test alone, whereas no false positives were expected when using the B–H correction.

RT-PCR Analysis

In order to validate microarray results, 24 identified genes were examined using a real-time RT-PCR approach. Nine hundred nanograms of total RNAs were reverse transcribed into cDNA using random primers according to the instructions from the RT^2 First Strand Kit (SA Biosciences, Frederick, MD). For the real-time PCR analysis, each 96-well plate contained 3 panels of the 24 selected target genes, 5 housekeeping genes, 1 reverse transcription control, and 1 positive PCR control (RT^2 Profiler PCR array system, SA Biosciences). The primer sets were designed and specifically optimized by a computer algorithm (SA Biosciences). PCRs were performed in triplicate on four cDNA preparations corresponding to four independent exposures per condition (sham, 6 or 24 h). Reactions were amplified using RT^2 SYBR Green qPCR Master Mix (SA Biosciences) in a MyIQ real-time PCR detection system (Bio-Rad, Hercules, CA). Fluorescence was measured at the beginning of each annealing/extension step. The relative expression of each mRNA was calculated by the ΔC_t method [Winer et al., 1999], and data were expressed as fold of induction, which corresponds to the ratio of exposed mRNA to sham mRNA.

RESULTS

Temperature Measurement and Heat Shock Response Analysis

The main known effect of exposure to MMW radiation is temperature rise. Therefore, we measured temperature dynamics within two central wells of the tissue culture plate at a distance of 1 mm from the bottom of the wells (Fig. 3A). The location of the optical fiber tip (Fig. 1B) corresponds to the maximal PD and to the peak of the temperature elevation within the plate. The data are shown for a 2 h period, which coincides with the most significant temperature rise rate. The steady-state was reached after 2 h of exposure, and the temperature increment was around 0.8–0.9 °C. The difference of 0.1 °C between two measurements may result from a slight misalignment of the antenna relative to the tissue culture plate's central axis. Using real-time RT-PCR, we verified that a 1 °C rise does not induce heat shock protein 70 and heat shock protein 27 (HSP70 and HSP27, respectively) expression in human keratinocytes (data not shown). These results confirm data from literature, showing that such a small temperature increase does not trigger a thermal biological response [Lovell et al., 2007]. This was confirmed by the DNA microarray analysis. Genes known to be sensitive to heat shock [Trinklein et al., 2004; Kultz, 2005] were extracted from microarray data and their overall expression was not modified in our exposure conditions (Fig. 3B). However, a detailed analysis shows that three genes are slightly but significantly induced after a 6 h exposure (Table 1). These genes, DNAJB1, DNAJB6, and HSPA1A, are induced by factors of 1.66, 1.44, and 1.93, respectively, which is not comparable to the classical overexpression induced by an acute heat shock (induction by factors of 38.7, 4.4, and 58.4, respectively; Trinklein et al.

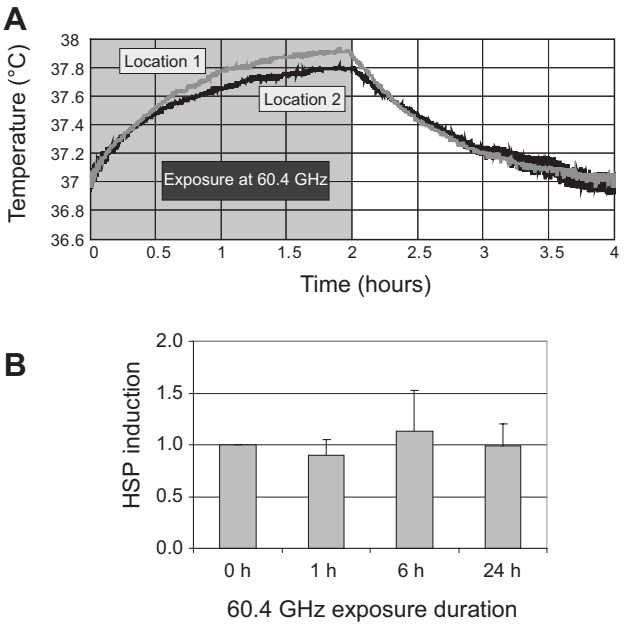

Fig. 3. **A**: Temperature dynamics of the cells exposed for 2 h at 60.4 GHz. **B**: Relative mRNA expression of heat shock proteins (HSP) listed in Table 1.

TABLE 1. Selection of Heat Shock Responsive Genes

Probe name	Genbank accession	Names	Gene symbol	Fluorescence intensity			
				Sham	1 h, 60.4 GHz	6 h, 60.4 GHz	24 h, 60.4 GHz
A_23_P90062	NM_006145	DnaJ (HSP40) homolog, subfamily B, member 1	DNAJB1	2759.1 ± 461.2	3221.0 ± 304.1; $P = 0.1532$	4592.2 ± 659.8; $P = 0.0051$	3680.8 ± 309.9; $P = 0.0196$
A_23_P51339	NM_007034	DnaJ (HSP40) homolog, subfamily B, member 4	DNAJB4	4T93.0 ± 208.9	389.9 ± 105.1; $P = 0.4234$	417.3 ± 206.2; $P = 0.6246$	310.7 ± 49.2; $P = 0.1787$
A_23_P385063	NM_058246	DnaJ (HSP40) homolog, subfamily B, member 6	DNAJB6	2248.9 ± 571.8	2286.2 ± 554.0; $P = 0.9284$	3247.0 ± 381.8; $P = 0.0319$	2110.6 ± 538.1; $P = 0.7366$
A_23_P36962	NM_006260	DnaJ (HSP40) homolog, subfamily C, member 3	DNAJC3	660.7 ± 86.7	667.8 ± 69.6; $P = 0.9030$	654.2 ± 106.6; $P = 0.9273$	657.2 ± 136.8; $P = 0.9672$
A_23_P56922	NM_002157	Heat shock 10 kDa protein 1 (chaperonin 10)	HSPE1	51152.4 ± 5050.5	46896.1 ± 6047.3; $P = 0.3227$	53488.8 ± 6330.7; $P = 0.5859$	53263.5 ± 10410.1; $P = 0.7323$
A_23_P257704	NM_001540	Heat shock 27 kDa protein 1	HSPB1	101588.7 ± 10850.3	98243.1 ± 15903.9; $P = 0.7416$	115443.5 ± 24584.7; $P = 0.3591$	118185.9 ± 35997.6; $P = 0.4331$
A_23_P161727	NM_001541	Heat shock 27 kDa protein 2	HSPB2	472.1 ± 177.4	430.1 ± 85.2; $P = 0.6896$	242.3 ± 69.5; $P = 0.0750$	381.4 ± 193.6; $P = 0.5157$
A_23_P92730	NM_006308	Heat shock 27 kDa protein 3	HSPB3	39.6 ± 25.4	20.0 ± 9.1; $P = 0.2230$	21.6 ± 18.9; $P = 0.3002$	36.1 ± 40.4; $P = 0.8878$
A_32_P25273	NM_002156	Heat shock 60 kDa protein 1 (chaperonin), nuclear gene encoding mitochondrial protein	HSPD1	87482.4 ± 7512.9	82295.1 ± 7995.6; $P = 0.3810$	85524.0 ± 11939.1; $P = 0.7923$	90468.0 ± 9636.0; $P = 0.6434$
A_23_P111132	NM_005345	Heat shock 70 kDa protein 1A	HSPA1A	17304.6 ± 5044.3	16259.0 ± 3934.5; $P = 0.7555$	33352.6 ± 5132.9; $P = 0.0043$	22733.5 ± 4639.7; $P = 0.1646$
A_23_P114903	NM_002155	Heat shock 70 kDa protein 6	HSPA6	633.4 ± 901.5	484.0 ± 222.5; $P = 0.7666$	945.2 ± 1019.2; $P = 0.6631$	392.0 ± 144.9; $P = 0.6319$
A_23_P162874	NM_005348	Heat shock protein 90 kDa alpha (cytosolic), class A member 1	HSP90AA1	30827.1 ± 2686.0	28169.0 ± 3809.8; $P = 0.3022$	35151.2 ± 2705.5; $P = 0.0638$	34819.8 ± 6936.2; $P = 0.3452$
A_23_P2601	NM_003299	Heat shock protein 90 kDa beta (Grp94), member 1	HSP90B1	77135.2 ± 13268.2	74982.8 ± 5785.3; $P = 0.7806$	68903.5 ± 8253.8; $P = 0.3401$	74669.3 ± 11607.9; $P = 0.7892$
A_23_P88119	NM_006644	Heat shock 105 kDa/110 kDa protein 1	HSPH1	13028.6 ± 4197.4	10582.6 ± 759.5; $P = 0.3300$	15132.4 ± 879.2; $P = 0.3936$	12734.4 ± 2643.3; $P = 0.9101$

Average of the fluorescent values (±SD) of four replicates of sham and exposed conditions. The *P*-values from paired *t*-test comparing sham and MMW exposures are indicated.

[2004]). Altogether, our data clearly show that the exposure conditions used in our experiments do not trigger a heat shock response in MMW-exposed keratinocytes.

Analysis of Microarray Data

After normalization of the expression levels, we selected 26,301 entities having fluorescence intensities higher than 50 for two RNA samples in at least one condition. Thereafter, the average ± standard deviation (SD) for the four replicates of sham and exposed genes was calculated. The results were filtered for FC >2, and the gene list was subjected to a *t*-test with a B–H correction (*P*-value cut-off of 0.05). No gene passed the statistical restrictions (Table 2), showing that exposure to MMW does not induce significant differential gene expression.

Because the B–H correction is a stringent test that can generate false negatives, we used a simple *t*-test to identify a list of genes possibly affected by MMW exposure. This approach allowed the identification of 130 transcripts potentially modulated by MMW exposure (Table 2 and Fig. 4). We found 111 genes downregulated and 19 upregulated. Interestingly, the short-term 1 h MMW exposure caused virtually no change in gene expression (1 gene, Fig. 4). Upon longer exposure (6 h), 120 gene entities were identified, whereas only 21 were found with a prolonged 24 h exposure. Therefore, most of the responsive genes were specific to the 6 h exposure, suggesting a transient and reversible cellular response. This might also reflect the temporal dynamics of the cell response, including early and late MMW responsive genes. These candidate genes are functionally widely diversified when classified by their Gene Ontology (GO) terms (data not shown). However, in absence of the B–H correction, we can expect that some false positives might contaminate our list of potential gene entities. So, to confirm the results from low stringency statistical analysis, real-time PCR analysis was performed on matched sample preparations (Fig. 2).

RT-PCR Validation

Quantitative real-time PCR can be considered as the most sensitive and reliable method for gene expression measurement, but this technology is not suitable for high-throughput studies. Thus, we selected 24 genes with the best combination of high FC and low *P*-value. PCR analysis of four independent exposures showed that five genes could be significantly confirmed as differentially expressed (Fig. 5). These genes are: (1) cysteine-rich protein 2 (CRIP2, *t*-test, $P = 0.001$ for a 6 h exposure), a zinc-binding protein involved in signaling, hematopoiesis, and cell proliferation; (2) Plexin D1 (PLXND1, *t*-test, $P = 0.031$ for a 6 h exposure), a transmembrane receptor involved in development; (3) Pentraxin-related gene (PTX3, *t*-test, $P = 0.009$ for a 6 h exposure), a protein involved in innate immunity and inflammatory response; (4) Serpin peptidase inhibitor (SERPINF1, *t*-test, $P = 0.038$ for a 6 h exposure), a secreted endopeptidase inhibitor that has anti-angiogenic and anti-proliferation functions; and (5) transient receptor potential cation channel (TRPV2, *t*-test, $P = 0.001$ for a 6 h exposure and $P = 0.003$ for a 24 h exposure), a calcium channel involved in sensory perception. Although not exactly identical, the decreases in mRNA levels detected by real-time PCR were close to those detected by microarrays (Table 3).

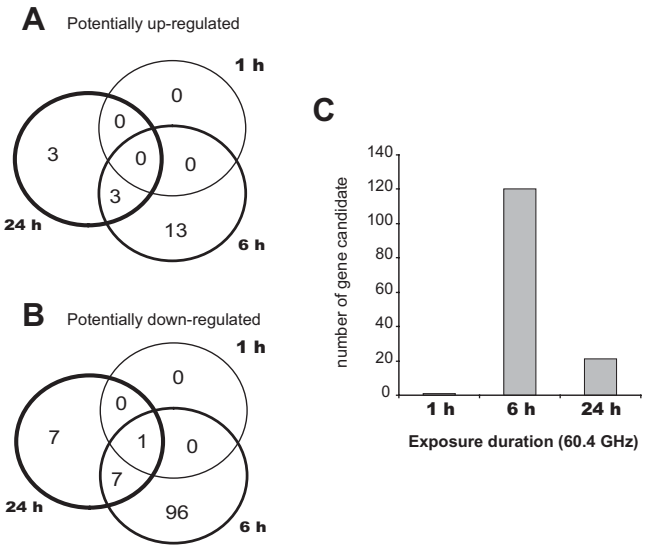

Fig. 4. Venn diagrams showing repartition of genes potentially upregulated (**A**), or potentially downregulated (**B**), after 1, 6, and 24 h of exposure. **C**: Number of candidate transcripts identified.

TABLE 2. Number of Transcripts Selected According to the Test Used

	FC > 2, *t*-test with B–H correction (*P*-value <0.05)	FC > 2, *t*-test (*P*-value <0.05)
1 h exposure versus sham (0 h)	0	1
6 h exposure versus sham (0 h)	0	120
24 h exposure versus sham (0 h)	0	21

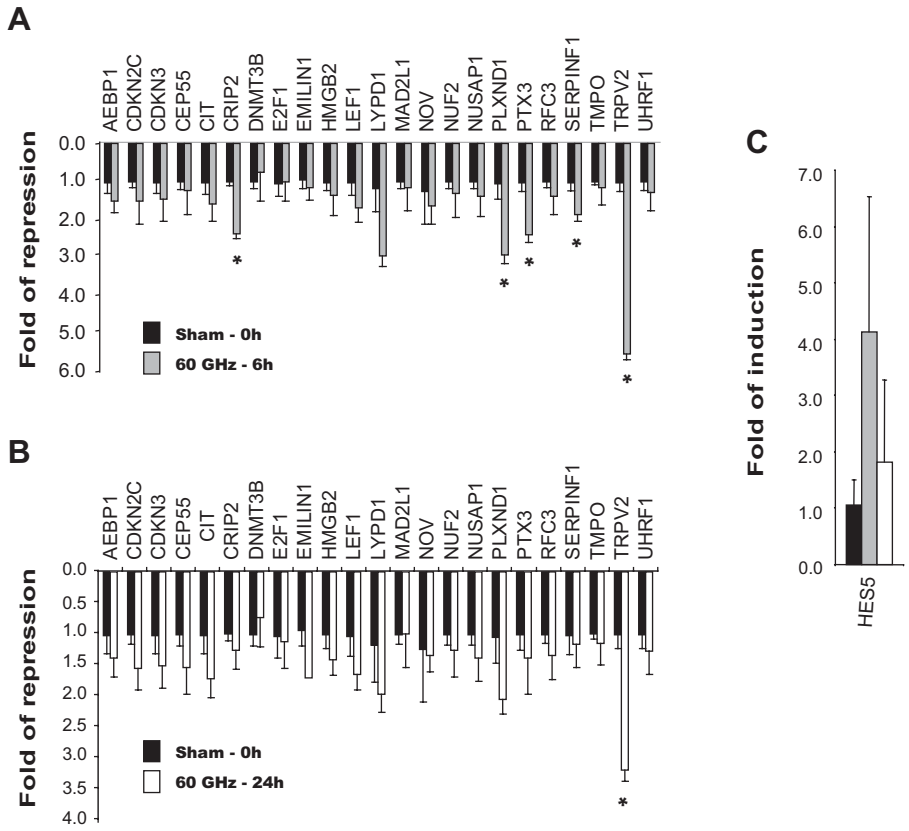

Fig. 5. Validation of microarray results using quantitative real-time RT-PCR. **A**,**B**: Downregulated genes. **C**: Upregulated genes. Each value is the mean ± SD of four independent experiments; $^{*}P < 0.05$ compared to control.

DISCUSSION AND CONCLUSION

In this study, we used gene expression analysis to investigate potential responses of human skin cells to low-power MMW radiation. Utilization of whole human genome microarrays permits a large-scale analysis without a priori preselection, and provides a wide picture of gene expression in the exposed cells. As change in gene expression is usually observed within a specific time frame, we exposed normal human keratinocytes for 1, 6, or 24 h to 60.4 GHz (with an average incident PD equal to 1.8 mW/cm^2). This strategy also reduces the possibility of masking effects due to compensation mechanisms.

Microarray data analysis using stringent statistical conditions showed no compelling evidence of gene modulation, demonstrating that MMW exposures with incident PD levels close to the exposure limits do not strongly modify gene expression. Using a less stringent analysis of microarray data, we identified a group of 130 transcripts potentially modulated by the MMW exposure. Some large-scale studies have already examined gene expression modification after microwave exposure (for a review, see McNamee and Chauhan [2009]). Most of these studies concern the effects of different mobile phone systems on various biological models. In addition, the data are very heterogeneous, making any comparison difficult. Several publications showed no significant difference between sham or exposed cells [Gurisik et al., 2006; Qutob et al., 2006; Whitehead et al., 2006a; Chauhan et al., 2007; Huang et al., 2008], or described weak changes that were not confirmed by RT-PCR validation [Zeng et al., 2006; Paparini et al., 2008; Roux et al., 2011; Sakurai et al., 2011], while other microarray studies reported modified gene expression after radiofrequency exposure [Belyaev et al., 2006; Nylund and Leszczynski, 2006; Remondini et al., 2006; Zhao et al., 2007; Huang et al., 2008]. However, these positive studies reported a small number of responsive genes (9–34) with a FC generally close to, or lower than 2. These genes belong to different functional families and data comparison does not permit establishing a list of common responsive genes. Regarding this aspect, our present work is not an exception because the group of 130 entities that

TABLE 3. MMW-Modulated Genes Confirmed by RT-PCR Analysis

Gene symbol	Description	Microarray data: Regulation	Microarray data: Fold change repression	RT-PCR data: Fold change repression	RT-PCR data: *P*-value (*t*-test)	Biological process (GO term)	Molecular function (GO term)	Cellular component (GO term)
CRIP2	Cysteine-rich protein 2 (Genbank Accession: NM_001312)	Down	At 1 h: **1.06** At 6 h: **2.46** At 24 h: **1.25**	— At 6 h: **2.33** At 24 h: **1.27**	— 0.001 0.248	Negative regulation of cellular process (GO:0048523); regulation of cell cycle (GO:0051726); negative regulation of progression through cell cycle (GO:0045786); RNA metabolic process (GO:0016070); regulation of transcription, DNA-dependent (GO:0006355)	Transcription factor activity (GO:0003700); DNA binding (GO:0003677); protein binding (GO:0005515)	Intracellular (GO:0005622); nucleus (GO:0005634); cytoplasm (GO:0005737); organelle (GO:0043226); plasma membrane (GO:0005886); intracellular membrane-bound organelle (GO:0043231); synapse (GO:0045202)
PLXND1	Plexin D1 (Genbank accession NM_015103)	Down	At 1 h: **1.11** At 6 h: **7.10** At 24 h: **1.92**	— At 6 h: **2.94** At 24 h: **2.49**	— 0.031 0.063	Developmental process (GO:0032502); organ morphogenesis (GO:0009887); angiogenesis (GO:0001525); cellular process (GO:0009987); signal transduction (GO:0007165)	Signal transducer activity (GO:0004871); transmembrane receptor activity (GO:0004888); protein binding (GO:0005515)	Intracellular (GO:0005622); membrane (GO:0016020); intrinsic to membrane (GO:0031224)
PTX3	Pentraxin-related gene, rapidly induced by IL-1 beta (Genbank accession NM_002852)	Down	At 1 h: **0.94** At 6 h: **2.90** At 24 h: **1.10**	— At 6 h: **2.38** At 24 h: **1.39**	— 0.009 0.386	Response to stress (GO:0006950); response to biotic stimulus (GO:0009607); immune response (GO:0006955), inflammatory response (GO:0006954); positive regulation of cellular process (GO:0048522); cellular metabolic process (GO:0044237); nitrogen compound metabolic process (GO:0006807); nitric oxide biosynthetic process (GO:0006809); vesicle-mediated transport (GO:0016192); positive regulation of endocytosis (GO:0045807); positive regulation of phagocytosis (GO:0050766)	Carbohydrate binding (GO:0030246); zymosan binding (GO:0001872)	Extracellular region (GO:0005576)
SERPINF1	Serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1 (Genbank Accession NM_002615)	Down	At 1 h: **1.01** At 6 h: **2.43** At 24 h: **1.22**	— At 6 h: **1.85** At 24 h: **1.16**	— 0.038 0.505	Developmental process (GO:0032502); organ morphogenesis (GO:0009887); cell differentiation (GO:0030154); cell proliferation (GO:0008283); nervous system development (GO:0007399); positive regulation of neurogenesis (GO:0050769); blood vessel development (GO:0001568); negative regulation of angiogenesis (GO:0016525)	Enzyme regulator activity (GO:0030234); serine-type endopeptidase inhibitor activity (GO:0004867)	Cytoplasm (GO:0005737); organelle (GO:0043226); melanosome (GO:0042470); cytoplasmic membrane-bound vesicle (GO:0016023); intracellular membrane-bound organelle (GO:0043231); extracellular region (GO:0005576)
TRPV2	Transient receptor potential cation channel, subfamily V, member 2 (Genbank Accession NM_016113)	Down	At 1 h: **1.22** At 6 h: **9.69** At 24 h: **2.74**	— At 6 h: **5.56** At 24 h: **3.23**	— 0.001 0.003	Neurological system process (GO:0050877); sensory perception (GO:0007600); response to temperature stimulus (GO:0009266); response to abiotic stimulus (GO:0009628); calcium ion transport (GO:0006816)	Channel activity (GO:0015267); passive transmembrane transporter activity (GO:0022803); cation transmembrane transporter activity (GO:0008324); metal ion binding (GO:0046872); calcium ion binding (GO:0005509)	Cytoplasm (GO:0005737); organelle (GO:0043226); melanosome (GO:0042470); cytoplasmic membrane-bound vesicle (GO:0016023); membrane (GO:0016020); intrinsic to plasma membrane (GO:0031226)

Gene Ontology (GO) terms were obtained from the Database for Annotation, Visualization and Integrated Discovery (DAVID). Fold change values are in bold font.

we identified also poorly overlapped with previously identified genes. For example, if we focus on MMW, our results differ from those reported by Millenbaugh et al. [2008], who identified 56 and 58 responsive genes, 6 and 24 h, respectively, after a 35 GHz exposure. This could be due to the presence of false positives in our list, or because of differences in biological models (rat skin vs. human primary keratinocytes) or exposure parameters (35 GHz at 75 mW/cm^2 vs. 60.4 GHz at 1.8 mW/cm^2). It is worthwhile to note that in Millenbaugh's study, the rat skin surface temperature exceeded 41 °C during exposure, whereas our experimental conditions had a moderate impact on temperature rise and did not induce a biological heat shock response from the cells.

RT-PCR validation showed that only 5 genes out of the 24 tested were confirmed to be differentially expressed after a 6 h exposure. Among these 5 candidates, 2 can be linked to the MMW bioeffects reported in the literature. PTX3 is involved in inflammatory responses, and during the past several years the effects of MMW irradiation on the immune system have been extensively studied [Rojavin and Ziskin, 1998; Makar et al., 2006]. PTX3 is a zymosan-binding protein, and it is noteworthy to mention that Gapeyev et al. [2008] have shown that MMW reduced both the footpad edema and local hyperthermia induced by zymosan in mice. TRPV2 is a widely expressed calcium channel. In neurons of the peripheral nervous system, TRPV2 is involved in heat sensing and has been proposed as a candidate for the mediation of MMW effects in pain therapy [Alekseev et al., 2010].

In conclusion, only five transcripts were found to be significantly affected. Compared to other microarray analyses studying the effect of pollutants or drug treatments, the number of responsive genes in the present study is extremely modest. Moreover, the gene expression modification is transient (mostly after 6 h of exposure) and with a limited amplitude (generally with a FC close to 2). One can only wonder about the consequence on human health, of such subtle changes at cellular level. Thus, we can consider that MMW (continuous wave at 60.4 GHz, 1.8 mW/cm^2) do not have any dramatic impact on primary human keratinocyte cultures. However, we cannot totally exclude that MMW could affect gene expression in vivo because skin is a complex tissue composed of different kinds of cells with various sensitivities. Moreover, one cannot exclude that other exposure conditions in terms of frequency, incident PD, polarization, or type of exposure (intermittent or continuous) could result in an amplified effect on cells. In this framework, the knowledge of the influence of electromagnetic field parameters on biological systems will become more important, taking into account the diversity of the emerging wireless technologies.

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