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Effects of different mobile phone UMTS signals on DNA, apoptosis and oxidative stress in human lymphocytes

Sachin Gulati, Pavol Kosik, Matus Durdik, Milan Skorvaga, Lukas Jakl, Eva Markova, Igor Belyaev*

Department of Radiobiology, Cancer Research Institute, Biomedical Research Center, Slovak Academy of Sciences, Bratislava, 845 05, Slovak Republic

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ABSTRACT

Different scientific reports suggested link between exposure to radiofrequency radiation (RF) from mobile communications and induction of reactive oxygen species (ROS) and DNA damage while other studies have not found such a link. However, the available studies are not directly comparable because they were performed at different parameters of exposure, including carrier frequency of RF signal, which was shown to be a critical for appearance of the RF effects. For the first time, we comparatively analyzed genotoxic effects of UMTS signals at different frequency channels used by 3G mobile phones (1923, 1947.47, and 1977 MHz). Genotoxicity was examined in human lymphocytes exposed to RF for 1 h and 3 h using complimentary endpoints such as induction of ROS by imaging flow cytometry, DNA damage by alkaline comet assay, mutations in TP53 gene by RSM assay, preleukemic fusion genes (PFG) by RT-qPCR, and apoptosis by flow cytometry. No effects of RF exposure on ROS, apoptosis, PFG, and mutations in TP53 gene were revealed regardless the UMTS frequency while inhibition of a bulk RNA expression was found. On the other hand, we found relatively small but statistically significant induction of DNA damage in dependence on UMTS frequency channel with maximal effect at 1977.0 MHz. Our data support a notion that each specific signal used in mobile communication should be tested in specially designed experiments to rule out that prolonged exposure to RF from mobile communication would induce genotoxic effects and affect the health of human population.

1. Introduction

For last few decades, the environment has been increasingly suffered from a new type of pollution created by electromagnetic radiations from wireless mobile communication (Blackman et al., 1979; Adey, 1993). This generated serious concerns regarding health of humans and safety of biota (Balmori, 2010). Different studies showed serious potential impact of electromagnetic radiation on our environment (Balmori, 2009; Lopatina et al., 2019). This electromagnetic pollution from mobile communication may affect not only human beings but also animals and birds (Balmori and Hallberg, 2007). In 2011, the International Agency for Research on Cancer (IARC), which is part of the World Health Organization, classified radiofrequency radiation (RF) including that from mobile phones as a possible carcinogen, group 2B (Baan et al., 2011). Long term mobile phone usage in different case control studies showed statistically significant association with increased risk of brain tumors (Wang and Guo, 2016; Bortkiewicz et al., 2017; Prasad et al., 2017; Yang et al., 2017).

As far as genotoxic effects are the most direct cause for carcinogenicity, available relevant studies were thoroughly reviewed in the IARC RF monograph (IARC, 2013). Diverse conclusions stemmed from these studies: in general, about half of studies found some RF genotoxicity (positive reports) while the other half have not (negative reports). This approximately similar numbers of positive and negative reports is in line with studies measuring some others biological endpoints of RF exposure (Huss et al., 2008; Apollonio et al., 2013; Cucurachi et al., 2013). While many studies on RF genotoxicity have been performed since the assessment of IARC in 2011, the balance between negative and positive studies did not change much (https://www.emf-portal.org/en/search/results?query=RF+genotoxicity&languageIds%5B%5D=en). However, results of all these studies are not directly comparable due to

* Corresponding author. Department of Radiobiology, Cancer Research Institute, Biomedical Research Center, Slovak Academy of Sciences, Bratislava, Slovak Republic.
E-mail address: igor.beliaev@savba.sk (I. Belyaev).

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dependence of the RF effects on a number of critical physical parameters of exposure, which vary significantly between studies (Belyaev, 2010; IARC, 2013). While specific absorption rate (SAR) and power flux density (PD) are the main determinants for the thermal RF effects, several other physical parameters of exposure including frequency, modulation, polarization, duration of exposure and also different biological variables have long been known to be critical for non-thermal RF biological effects such as induced by exposure to various sources of mobile communication (Blackman, 1992, 2009; Adey et al., 1999; Belyaev et al., 2000).

Free radicals are a group of highly reactive molecules having unpaired electrons in the outer orbit. Most known free radicals are reactive oxygen species (ROS) derived from oxygen metabolism. Upon overproduction, these reactive species can damage various molecules including DNA leading to increased mutations, changed cell death and cell growth, and thus contributing to the multistage carcinogenesis process. About 90% of available studies have reported that RF exposure causes oxidative stress as revealed by increase in ROS, oxidized proteins, peroxidized lipids and fragmented DNA, see for review (Georgiou, 2010; Yakymenko et al., 2015). However, the relevance of RF-induced ROS to DNA damage was less investigated and some studies reported that RF-induced ROS was not followed by DNA damage (Durdik et al., 2019).

It has been suggested that oxidative stress could be a key factor for RF-related incidence of brain tumors and childhood leukemias (De Iuliis, Newey et al., 2009). However, no studies are available to test whether mutations related to brain tumors and childhood leukemias are induced by exposure to RF signals used by mobile phones. In particular, different frequency channels of UMTS signals used in 3G technology have not been tested so far.

Tumor suppressor gene TP53 encoding p53 protein is the most commonly mutated gene in human cancers including brain tumors (Kandoth et al., 2013; Bouaoun et al., 2016). Somatic TP53 mutations have been detected in up to 20% of acute myeloid leukemia (AML), often associated with a complex karyotype, resulting into inferior survival rates (Grossmann et al., 2012; Rucker et al., 2012). Recent data suggested that somatic TP53 mutations may represent early leukemogenic events, possibly by initiating mutations acting as mediators of resistance in this type of leukemia (Lal et al., 2017).

Other early primary genetic abnormalities in the origination of acute childhood leukemia are chromosomal translocations in hematopoietic cells resulting in so-called preleukemic fusion genes (PFG). Two chromosomal translocations with corresponding PFG are frequent in pediatric acute lymphoid leukemia (ALL): t(12;21) (p13;q22) TEL-AML1 (24-26%) and t(4;11)(q21;q23) MLL-AF4 (~5%). MLL-AF9 belongs to the most frequent PFG for acute myeloid leukemia (AML). In this study, for the first time, we applied several complementary techniques to validate whether exposure of human lymphocytes to RF at different UMTS frequency channels induce ROS, DNA damage, apoptosis, TP53 mutations and most frequent PFG.

2. Materials and methods

2.1. Chemicals

Reagent grade chemicals were obtained from Sigma (St. Louis, MI, USA) and Merck (Darmstadt, Germany).

2.2. Ethical considerations

The Ethics Committee of Children's Hospital in Bratislava has approved this study. All UCB samples were provided with an informed consent from a parent for study participation.

2.3. Cells

In vitro cultures of human lymphocytes were used to investigate the effect of RF exposure. Cells were isolated from UCB and cell aliquots were cryopreserved in liquid nitrogen by Dr. M. Kubes (Eurocord, Slovakia) as described before (Vasilyev et al., 2013). Each sample was thawed in a water bath and diluted in Roswell Park Memorial Institute (RPMI) media supplemented with 10% Fetal Bovine Serum (FBS), and 1% antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin). After removal of adherent cells (mostly monocytes) by 1 h incubation in culture flasks, the remaining lymphocytes were subjected to RF exposure.

2.4. RF exposure

We used the RF exposure unit based on UMTS test mobile phone (model 6650, Nokia, Helsinki, Finland), output being 0.25 W, and transverse electromagnetic transmission line cells (TEM-cells) as previously described (Belyaev et al., 2009; Durdik et al., 2019). Phoenix software (Nokia, Helsinki, Finland) was used to control parameters of exposure.

As far as available data indicate dependence of the non-thermal RF effects on carrier frequency (Belyaev, 2010; Belyaev, 2015), different UMTS frequency channels were tested. Each channel represented a 5 MHz wide frequency band with the middle frequency of 1923, 1947.47, or 1977 MHz. RF exposure and sham exposure was performed simultaneously for 1 and 3 h in a humidified CO_2 incubator at 5% CO_2 and 37 °C (Heracell 150i, Thermo Fischer Scientific, Waltham, Massachusetts, USA) in two identical TEM-cells. Cells were exposed in 14 ml round-bottom tubes (Sarstedt, Numbrecht, Germany), 5 ml of cell suspension at concentration of 2×10^6 cells/ml in each tube. Standard UMTS modulation Quadrature Phase Shift Keying (QPSK) was used. The specific absorption rate (SAR) was determined by measurements and numerical calculations using the finite different time domain (FDTD)-method as comprehensively described elsewhere (Sarimov et al., 2004) (Belyaev et al., 2009). The obtained SAR of 40 mW/kg was much lower than the currently accepted value for mobile phones (2 W/kg). Our UMTS signals, including frequencies and modulations, were those ordinary used by 3G mobile phones and SAR values were in the range of those exposing people during ordinary mobile phone calls. Taking into account all possible uncertainties, the SAR values at all locations within the RF exposed samples were always well below any measurable thermal effect. We measured temperature of samples before and after exposures with a precision of 0.1^0C and didn't find any changes. Static magnetic field (SMF) at the locations of real and sham UMTS exposures was 37 μT and background extremely low frequency (ELF) magnetic field was not more than 0.1 μT rms. As a positive control, we used 1 h treatment with tert-Butyl Hydroperoxide (TBHP) at 20 μM or 200 μM.

2.5. Alkaline comet assay

Alkaline comet assay also known as single cell gel electrophoresis (SCGE) was performed according to Singh et al., (1988) and Tice et al., (2002) with minor modifications. Slides were prepared in duplicates. Briefly, 1×10^5 lymphocytes (20 μl) were mixed with 80 μl of warm 0.5% low melting agarose prepared in phosphate buffer saline (PBS) (0.02% KCl; 0.8% NaCl; 0.29% $Na_2H_3PO_4$ x $12H_2O$; 0.02% KH_3PO_4 in deionized water) and this mixture was layered as a second layer on slides precoated with 1% normal melting agarose and stored at 4 °C for 15 min. The slides were treated for 1 h in freshly prepared, chilled lysis buffer solution (25 mM NaCl, 100 mM sodium EDTA, 10 mM Tris, 1% Triton X −100, 10% DMSO, pH adjusted to 10) at 4 °C. Then slides were incubated in alkaline

electrophoresis buffer (10 N NaCl, 200 mM EDTA, pH adjusted to 13) for 40 min followed by electrophoresis (0.67 V/cm) for 30 min in the same buffer. The slides were then neutralized with Tris buffer (0.4 M Tris, pH adjusted to 7.5), rinsed with distilled water, and stained by ethidium bromide (5 μg ml^{-1}) before analysis. A total of 100 cells from each of the duplicate slides were examined randomly by the Zeiss Axioscope 2 epifluorescence microscope (Carl Zeiss Microscopy, Jena, Germany). Comet assay results were analyzed as tail moment (TM), the product of the tail length and the tail intensity, using the Metafer software (Metasystems, Altlussheim, Germany).

2.6. Reactive oxygen species

ROS were analyzed using Cell ROX Green kit (Life technologies, New York, USA) as previously described (Durdik et al., 2017). Briefly, 2 μl of 2.5 mM Cell ROX solution was added directly to 500 μl of cell suspension in concentration 1×10^6/ml immediately after exposure or sham exposure. Then antibody against white blood cells (including lymphocyte) surface marker was added, specifically, 2 μl CD45-V450 conjugate (BD biosciences, San Jose, California, USA) along with 3 μl of 7-AAD (BD biosciences) for staining nonviable cells. After incubation for 45 min in the CO_2 incubator, the samples were analyzed by imaging flow cytometer (ImageStreamX-100, Amnis-Luminex) and IDEAS software (Amnis Corporation, Seattle, WA, USA). Compensation matrix was created by the compensation wizard in the FACS Diva software (BD Biosciences, San Jose, CA, USA) after acquisition of single color stained samples and unstained control.

2.7. Apoptosis

Cells were harvested immediately and 24 h after exposure and apoptosis was analyzed as previously described (Durdik et al., 2017) simultaneously with ROS measurements. Briefly, 5×10^5 cells were spun down (100 g/10 min), washed with PBS and resuspended in 100 μl of the Annexin kit buffer (Roche, Basel, Switzerland). Cells were then stained with Annexin-V (Roche, Basel, Switzerland), 7AAD (BD biosciences) and anti-human CD45-V450 (BD biosciences) for white blood cell staining. The percentage of live (Annexin-V negative, PI negative), early apoptotic (Annexin-V positive, PI negative) and late apoptotic/necrotic (LAN) (Annexin-V positive, PI positive) cells was assessed using BD FACS Canto II flow cytometer (BD biosciences). Where LAN cells were more abundant, compensation were performed on samples. Single color stained tubes were acquired and compensation were generated automatically by BD FACS Diva software.

2.8. RNA/DNA isolation and cDNA synthesis

RNA for analysis PFG was isolated from 2.5×10^6 cells immediately after the end of 1-h and 3-h RF exposure from 1977 MHz frequency with innuPREP DNA/RNA mini Kit (Analytik Jena, Germany). cDNA was synthesized by reverse transcription in the standard reaction containing 1 μg of total RNA as we previously described (Skorvaga et al., 2014). At the same time, DNA for analysis of TP53 gene mutation was isolated from 1.5×10^6 cells using DNAzol genomic DNA isolation reagent following manufacturer protocol (Molecular Research Center, Ohio, USA).

2.8.1. RSM assay

Restriction site mutation (RSM) assay detects point mutations at restriction enzyme sites in TP53 gene as loss of p53 function (Bates and Vousden, 1999). The RSM assay was performed according to (Morgan et al., 2003) with some modifications. Digestion of genomic DNA was performed in 15-μl reaction volume with 1 μl of highly efficient Anza restriction endonuclease (Thermo Fisher Scientific, Waltham, MA, USA) overnight at optimal temperature, followed by additional digestion with 1 μl for 2 h. PCR contained 0.1 mM each dNTP's (Thermo Fisher Scientific), 300 nM each forward and reverse primers (Sigma Genosys, St. Louis, MI, USA), 1 μg of double-digested DNA and 2.5 U Dream*Taq* DNA polymerase (Thermo Fisher Scientific) in 1x TK buffer (20 mM Tris.Cl, pH 8.5; 50 mM KCl) with 3 mM $MgCl_2$ final concentration. The PCR products were purified by ethanol precipitation and re-digested with 1 μl Anza restriction enzyme (Thermo Fisher Scientific) in 20-μl volume for 3 h. One half of re-digested PCR product was analyzed by 2% agarose gel electrophoresis with RedGel stain (Biotium, Fremont, CA, USA) present in the gel using 0.5x TBE running buffer (Serva, Heidelberg, Germany). The gels were photographed with Gel documentation system MiniBis Pro (DNR-Imaging Systems Ltd., Neve Yamin, Israel). Second half of re-digested PCR product was saved for cloning/sequencing in case when the mutation containing fragment was detected.

2.8.2. Analysis of PFG by real-time quantitative PCR

RT-qPCR was performed as was previously described (Skorvaga et al., 2014; Kosik et al., 2017) using AriaMX real-time PCR system (Agilent Technologies, USA). The protocol, primers and probes were designed according to Gabert et al., (2003). Frequently occurring ALL/AML-associated PFG were tested, namely: TEL-AML1, MLL2-AF4, and MLL-AF9. The samples were run in triplicate and regarded as positive if at least one reaction was tested positive.

2.9. Statistical analysis

Mean and standard deviation (SD) were computed for the scores and the statistical significance of effects were determined using analysis of variance (ANOVA) adjusted for multiple comparisons using post-hoc tests such as Fisher LSD or Scheffe test with Statistica software (Dell software, Round Rock, Texas, USA). Differences were considered statistically significant at the value of $p < 0.05$.

3. Results

3.1. DNA damage

UCB cells from three different probands were exposed to UMTS RF at different frequencies (1923, 1947.47, or 1977 MHz) for 1 h and 3 h. Upon RF exposure, DNA damage was analyzed by alkaline comet assay. The representative photomicrographs of cells with damaged DNA are shown in Fig. 1A & B. Blood lymphocytes are known to be very sensitive to apoptosis, for instance induced by regular freeze and thaw process. Apoptotic cells were differentiated from viable cells according to the appropriate guidelines (Fig. 1C) and were not analyzed for DNA damage by the comet assay. TBHP treated cells have shown statistically significant increase in the TM compared to the untreated cells (ANOVA, $p < 0.001$). The TMs measured in cells after RF exposure are shown in Table 1. Analysis of data by multifactorial ANOVA has shown statistically significant dependence of the tail moment on RF exposure ($p = 0.04$). However, no dependence on exposure duration was revealed providing possibility for pooling the data for 1 and 3 h. The RF effect was also observed as a higher tail moment in the samples exposed at the 1977 MHz frequency if the data from 1-h and 3-h exposures were pooled ($p = 0.04$). Further analysis of the 1977 MHz effects split according to the duration of exposure did not show higher TM in the exposed samples. Analysis of pooled or split data at other frequencies, 1923 or 1947.47 GHz, did not show statistically significant effect of RF exposure on DNA damage. Summarizing the results, we

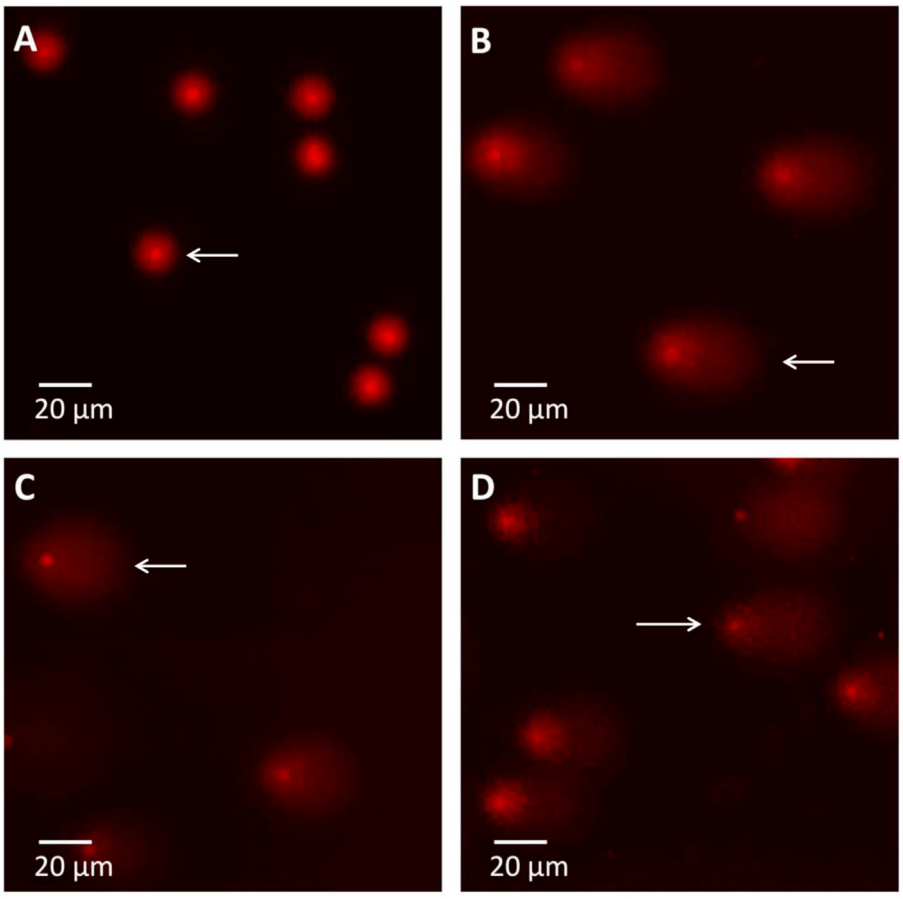

Fig. 1. Representative images of apoptotic cells and viable cells with and without DNA damage. A) Undamaged round-shape viable cells without DNA tails; B) DNA-damaged cells that contain a DNA tail and a head like a comet; C) late apoptotic cell with severely fragmented DNA around a small head; D) Severely DNA-damaged comet cells after treatment with TBHP.

Table 1
The data on alkaline comet assay for different frequencies of RF exposure.

Frequency (MHz)	Exposure duration (h)	Exposure	Tail moment (µM) (Mean ± SD)	P-value
1923.0	1	Exposed	3.79 ± 0.79	0.32
		Sham	2.86 ± 1.02	
	3	Exposed	3.94 ± 2.07	0.20
		Sham	2.71 ± 1.18	
1947.47	1	Exposed	6.07 ± 2.47	0.16
		Sham	4.72 ± 0.64	
	3	Exposed	4.34 ± 0.81	0.85
		Sham	4.52 ± 1.31	
1977.0	1	Exposed	2.12 ± 0.47	0.49
		Sham	1.84 ± 0.13	
	3	Exposed	2.71 ± 0.52	0.42
		Sham	2.03 ± 0.52	
Positive control	1	TBHB	25.53 ± 5.63	<0.001

Data from experiments with cells from three probands are shown. Differences between exposed and sham-exposed samples were defined by the univariate ANOVA followed by the post hoc Fisher LSD test.

observed relatively low but statistically significant effect of RF exposure on DNA damage in lymphocytes indicating dependence of this effect on the frequency of the UMTS signal.

3.2. Reactive oxygen species

As far as our comet assay results indicated that the effect of RF exposure could be frequency dependent, we exposed cells from 3 probands to UMTS for 1 and 3 h at the frequencies of 1923 and 1977 MHz and analyzed ROS and percentage of live $CD45^+$ lymphocytes immediately and also 24 h after 3-h exposure by the imaging flow cytometry (ImageStream X-100). Representative images of cells are shown in Fig. 6 (Supplementary Data) and obtained data in Figs. 2 and 3. Multifactorial ANOVA showed effect of neither RF exposure ($p = 0.36$), nor time of exposure/sham exposure ($p = 0.25$). By further analysis, we didn't find any difference in ROS

between RF exposed and sham groups for all analyzed time points (ANOVA with Scheffe post-hoc, see Table 3, Supplementary data). As a positive control we used treatment with 200 μM TBHP that significantly induced ROS compared to the RF exposed/sham exposed samples (*t*-test, $p < 0.001$) (Table 3, Supplementary data).

Analysis of cell survival by staining with 7-AAD showed neither effect of UMTS exposure ($p = 0.94$) nor influence of frequency ($p = 0.12$) on cell viability. As expected, cell survival decreased with the time of incubation ($p < 0.001$), but the observed decrease in cell viability was caused by the endogenous apoptosis, not by the UMTS exposure.

3.3. Apoptosis

Using flow cytometry, we further analyzed apoptosis in cells from 3 probands immediately after 1-h and 3-h RF exposure at the frequency of 1977 MHz and also after 24-h incubation of the RF exposed samples (Table 4, Supplementary data). Representative figure of gating strategy to discriminate live cells, early apoptotic cells and LAN cells is shown in Fig. 7 (Supplementary data). Multifactorial ANOVA of pooled data showed significant dependence of cell viability on incubation time ($p < 0.001$). Similar decrease in cell viability was observed at 24 h compared to 3 h in both sham ($p = 0.02$) and RF exposed group ($p = 0.03$) suggesting endogenous nature of apoptosis in these cells. Indeed, RF exposure of cells did not result in any effect ($p = 0.95$) (Fig. 4). These data were in line with the results obtained by measuring 7-AAD LAN cells with imaging flow cytometry. We can conclude that UMTS exposure under chosen conditions did not induce apoptosis in lymphocytes. We didn't find any effect on apoptosis also in our previous study with 1947.47 MHz frequency (Durdik et al., 2019).

3.4. Mutational analysis of TP53 gene

We performed three experiments with cells of different probands to analyze whether UMTS exposure at 1977 MHz induces mutations in selected mutation hotspots of *TP53* gene, namely codon 175 (exon 5) and codon 213 (exon 6). Exposed and sham lymphocytes were collected immediately after 1-h and 3-h RF exposure and then 24-h post-exposure. In Fig. 5, the photo of a representative gel is shown. The size of mutated, i.e. digestion-resistant DNA fragment was expected to be 188 bp, in contrast to fully digested PCR product yielding two fragments, 114 bp and 74 bp, respectively. The gel is highly overexposed in order to increase chance to visualize the mutated band. No mutations were detected at all tested conditionals of UMTS exposure using the RSM assay with sensitivity of $10^{-4} - 10^{-5}$.

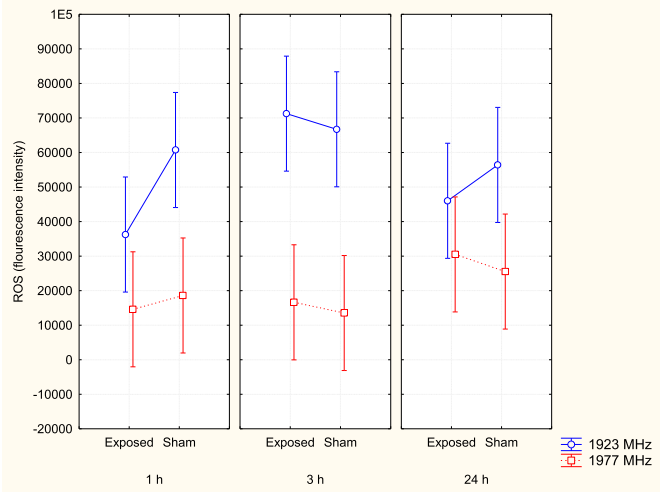

Fig. 2. ROS for different carrier frequencies and durations of UMTS RF exposure, imaging flow cytometry. The data from three experiments with cells from different probands are shown in each data point. Error bars show 95% confidence interval.

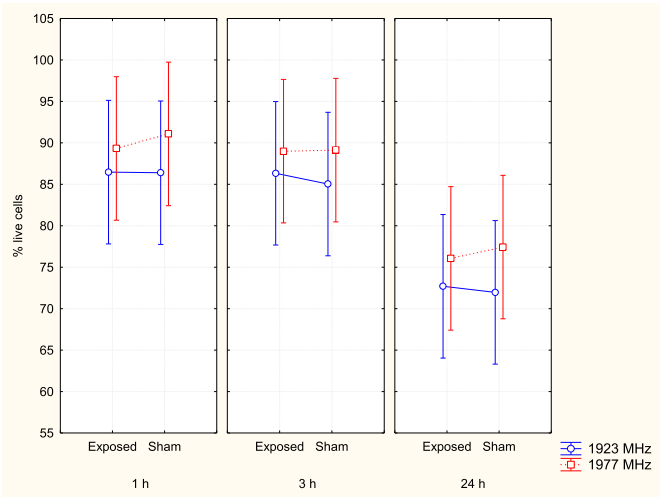

Fig. 3. Cell viability after UMTS RF exposure at different carrier frequencies and exposure durations as measured by imaging flow cytometry. The data from experiments with cells from three probands are shown. Error bars show 95% confidence interval.

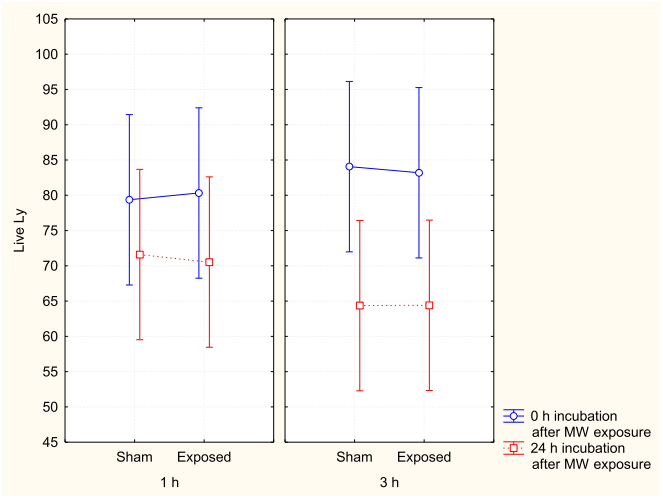

Fig. 4. Cell viability measured by FACS after different durations of RF exposure. The data from experiments with cells from three probands are shown. Error bars show 95% confidence interval.

3.5. Preleukemic fusion genes

We used UCB cells from three different probands to test possible induction of PFG by RF exposure at the 1977 MHz frequency. TEL-AML1, MLL2-AF4 and MLL1-AF9 preleukemic fusion genes, which are associated with ALL and AML, respectively, were analyzed by the RT-qPCR. We found TEL-AML1 positivity in three out from seven sham exposed samples but only in one out from seven UMTS exposed samples (Table 2). MLL2-AF4 fusion gene was found in two out from seven exposed/sham exposed. We did not observe MLL-AF9 PFG in any of the tested samples. All positive observations were characterized by very low number of PFG copies about 2.6

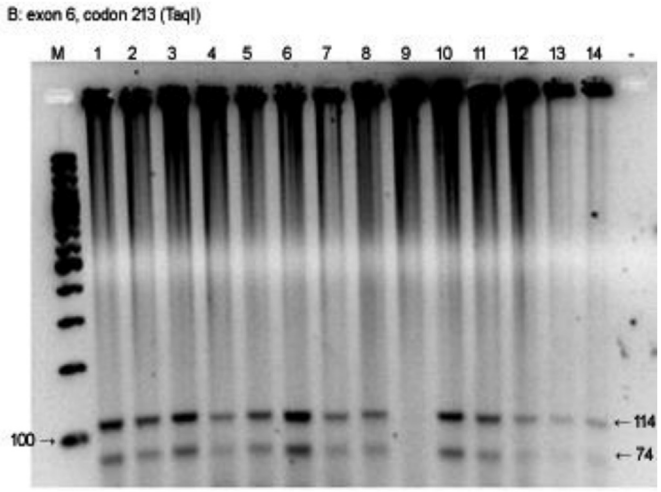

Fig. 5. Representative gel from the RSM experiment involving the TaqI restriction site of codon 213. M – 100 bp DNA ladder, 1–12: P377, P367, and P320 - sham 1 h, UMTS 1 h, sham 3 h, UMTS 3 h, respectively, 13 and 14: P367 sham 24 h and UMTS 24 h, respectively, - negative control (no DNA template in PCR). Two bands, corresponding to 114 bp and 74 bp, result from a complete digestion of exon 6, codon 213, in TaqI T↓CGA restriction site indicating that the mutation in this hot spot site of p53 gene was not introduced. In case of introducing a mutation in this site, a 188 bp band would appear.

copies per 10^5 cells showing that this positivity was at the level of sensitivity of the applied RT-qPCR method. Taking into account this fact and sporadic nature of the observed PFG positivity, we concluded that UMTS RF exposure at the 1977 MHz frequency did not induce the tested PFG. Additionally we compared the RNA expression as the yield of RNA per cell after the UMTS and sham exposure. Analysis by multifactorial ANOVA has shown significant effect of UMTS exposure on RNA expression ($p = 0.03$). By further analysis by one-way ANOVA followed by Fisher LSD we found significant reduction of RNA in cells upon 1-h UMTS exposure ($p = 0.03$).

4. Discussion

In the present investigation, we analyzed non-thermal effects of RF from 3G mobile phone at different UMTS frequency channels on human lymphocytes. We used complimentary biomarkers to assess DNA damage by alkaline comet assay, ROS by imaging flow cytometry, apoptosis by flow cytometry, p53 mutations by RSM method, and induction of PFG by RT-qPCR method. Comet assay is a very sensitive technique which can detect damage in DNA at single cell level and widely accepted in genonotoxicity studies worldwide (Garaj-Vrhovac et al., 2002; Guerci et al., 2011; Seidel et al., 2012). Thus, we used comet assay to detect eventual damage caused by different UMTS frequency channels, 1923, 1947.47, and 1977 MHz. Analysis of data by multifactorial ANOVA has shown statistically significant dependence of the comet tail moment on RF exposure but not on duration of exposure. This RF effect was also observed as a higher TM in the samples exposed at the 1977 MHz frequency if the data from 1-h and 3-h exposures were pooled. Analysis of data at other frequencies, 1923 or 1947.47 GHz, did not show statistically significant effect of RF exposure on DNA damage. Thus, we found relatively small but statistically significant induction of DNA damage in dependence on UMTS frequency channel with maximal effect at 1977 MHz. Our comet assay findings are in line with some previous studies (Lai and Singh, 1996, 1997; Garaj-Vrhovac and Orescanin, 2009; Shahin et al., 2013; Gulati and Yadav, 2016) although other studies are in contrast with our data (Sun et al., 2006a, 2006b; Hintzsche and Stopper, 2010; Juutilainen et al., 2011).

Of note, the data from different studies cannot be directly compared due to dependence of the non-thermal RF effects on several biological and physical variables, which significantly vary between studies (Belyaev, 2010; IARC, 2013). In particular, dependence of the non-thermal RF effects on frequency has previously been reviewed (Pakhomov et al., 1998; Belyaev et al., 2000). Frequency-dependent interactions of RF with such targets as cellular membranes, chromosomal DNA, free radicals, proteins and ions in protein cavities may be involved in such effects of RFs (Ismailov, 1987; Chiabrera et al., 2000; Binhi, 2002; Belyaev, 2015).

Cell type was also critical as far as different cell types reacted significantly differently to the same non-thermal RF exposure (Belyaev, 2010; IARC, 2013). So far, very few studies have analyzed genotoxicity of the UMTS signals in human lymphocytes by alkaline comet assay. Sannino et al., (2006) exposed blood leukocytes to 1950 MHz frequency used by UMTS mobile communication for 24 h and didn't find any DNA damage. Ivancsits et al., (2005) also investigated the effect of UMTS exposure using different cell types and didn't reveal any genotoxic effects in lymphocytes. El-Abd et al. (El-Abd and Eltoweissy, 2012) reported time dependent DNA damage in human lymphocytes from UMTS exposure. Intermittent exposure to UMTS as compare to continuous wave exposure resulted in significant effects in fibroblasts but lymphocytes didn't show the same pattern in the study by Schwarz et al., (2008). Al-Serori et al., (2018) studied the role of serum in media with different cell types including lymphocytes for the effects of UMTS exposure. Although DNA damage was serum dependent in glioblastoma cells, no DNA damage was found in lymphocyte by UMTS exposure with or without serum. In line with our results, recent study by Bektas et al. found a link between mobile phone exposure during pregnancy and DNA damage measured with comet assay in UCB lymphocytes (Bektas et al., 2020).

As already mentioned above, results of all these comet assay studies are not directly comparable due to using different biological and physical variables and strong dependence of the RF effects on

Table 2
Expression of MLL2-AF4 and TEL-AML1 preleukemic fusion genes after 1-h and 3-h UMTS exposures as measured by RT-qPCR in cells of three different probands. Number of PFG positive samples out from three tested samples is shown.

	P377 SHAM 1h	P377 UMTS 1h	P377 SHAM 3h	P377 UMTS 3h	P367 SHAM 1h	P367 UMTS 1h	P367 SHAM 3h	P367 UMTS 3h	P320 SHAM 1h	P320 UMTS 1h	P320 SHAM 3h	P320 UMTS 3h
RNA (ng/μl)	149.3	84.2	114.6	97.5	154.7	138.6	172.1	141.1	141.4	98.8	112.1	131.9
Yield (pg/cell)	1.79	1	1.38	1.17	1.66	1.5	1.84	1.5	1.77	1.24	1.2	1.41
c-ABL copies/10^5	36	11.55	27.476	16.55	24.46	12	19.181	20	4.445	12.458	26.236	5.09
MLL2-AF4	1/3	0/3	0/3	1/3	0/3	0/3	0/3	0/3	1/3	0/3	0/3	1/3
TEL-AML1	0/3	0/3	1/3	0/3	0/3	0/3	1/3	1/3	0/3	0/3	1/3	0/3
MLL1-AF9	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

these variables (Belyaev, 2010). Given such dependence, it should be concluded that RF exposure may or may not affect DNA damage and repair strongly dependent on exposure conditions. In particular, the frequency/frequency channel is of importance as shown previously for GSM (Markova et al., 2005; Belyaev et al., 2009) and now for UMTS mobile phone. Along with other available data on dependence of the non-thermal RF effects on frequency (Belyaev, 2010; IARC, 2013), the obtained here data suggest that each signal for mobile communication should be tested in specially designed experiments before being used in mobile communication.

Except for frequency, apparently controversial findings stemming from different studies on RF-induced DNA damage can be accounted for several other experimental conditions. In particular, background ELF EMF and SMF were consistently reported to affect response to RF exposure (Belyaev, 2010; IARC, 2013), although remain either different or unreported in majority of studies. Thus, background ELF EMF and SMF (0.2 μT and 37 μT in our study) may be one of the reasons underlying eventual inconsistency (Blackman, 2009; Durdik et al., 2019).

Despite significant progress, there is still substantial lack of knowledge in biophysical modeling of RF induced non-thermal biological effects, which would predict effective and respectively inefficient conditions of RF exposure (Belyaev, 2015). A significant number of studies reviewed in (Georgiou, 2010, Yakymenko et al., 2016) suggested the role of oxidative stress (excessive formation of ROS) in RF induced DNA damage. It is generally accepted that stimulation of oxidative stress can generate DNA damage (Moustafa et al., 2001; Stopczyk et al., 2005; Blank and Goodman, 2011; Burlaka et al., 2013; Gulati et al., 2018) and apoptosis (Desai et al., 2009; Shahin et al., 2015).

Lu et al. reported that apoptosis was induced by RF exposure through the mitochondrial pathway mediated by activating ROS and caspase-3, and decreasing the mitochondrial potential (Lu et al., 2012). Friedman et al., studied the link between RF exposure and cancer through ERK-MAPK signaling pathway and found that RF-induced ROS activates ERK cascade by stimulating matrix metalloproteinase (Friedman et al., 2007). Several studies suggested that ROS plays an important role in cell death and signal transduction induced by non-ionizing radiations (De Iuliis, Newey et al., 2009; Kesari et al., 2013; Furtado-Filho et al., 2014). Of note, ROS level may be only temporarily induced by RF expsure due to subsequent activation of the antioxidant defense mechanism (Marjanovic et al., 2015; Durdik et al., 2019). Thus, here we analyzed ROS and apoptosis in UCB lymphocytes upon exposure to UMTS RF. ROS were measured by imaging flow cytometry immediately and 24 h after exposure at two carrier frequencies (1923 and 1977 MHz) for different time durations, 1 and 3 h. Multifactorial ANOVA analysis showed effect of neither RF exposure nor duration of exposure/sham exposure and incubation time. By further analysis, we didn't find any difference in ROS between RF exposed and sham groups for all analyzed time points (ANOVA with Scheffe post-hoc). Of not, exposure at one of these frequencies, 1977 MHz, resulted in weak but statistically significant induction of DNA damage as measured with alkaline comet assay while another frequency, 1923 MHz, was tested ineffective. Vice versa, UMTS exposure at 1947 MHz induced ROS level in identical experiments (Durdik et al., 2019) while did not induce DNA damage as measured in this study. Lack of relationship between induction of ROS and DNA damage revealed in this study may be accounted for adaptive reaction of cells to oxidative stress resulting in time dependent kinetics of ROS, which may be increased at other time points as analyzed in this study. Other possible mechanisms for induction of DNA damage, which do not involve ROS production, deal with impact through RF-induced changes in molecular conformation (Chiabrera et al., 2000; Matronchik and Belyaev, 2008). According to these mechanisms, RF may either affect availability of DNA to DNA-breaks, which are physiologically induced by enzymes such as topoisomerases and endonucleases, or increase activity of these enzymes affecting binding of their active centers with divalent ions such as Zn, Ca, and Mg. We also analyzed apoptosis/cell viability by imaging flow cytometry in the same experiments with ROS but no effect of RF exposure was found. In further experiments we tested by standard flow cytometry whether UMTS exposure at the frequency of 1977 MHz, which was shown to induce DNA damage, also induced apoptosis in UCB lymphocytes. The obtained data solidified our conclusion stemming from analysis by imaging flow cytometry that UMTS exposure did not induce apoptosis in lymphocytes.

While DNA damage is a prerequisite for formation of mutations, it can be efficiently repaired during DNA damage response. Of specific interest are mutations in those genes, which are involved in origination of various types of cancer. TP53 encoding p53 protein is the most commonly mutated gene in human cancers including brain tumors and leukemia. Preleukemic fusion genes TEL-AML1, MLL-AF4 and MLL-AF9 are most frequent in pediatric acute lymphoid leukemia and acute myeloid leukemia, respectively. Thus, we analyzed mutations in *TP53* gene by the RSM method and induction of the aforementioned PFG by the RT-qPCR. No mutations in selected *TP53* gene mutation hotspots were detected with relatively high sensitivity (10^{-4} to 10^{-5}). Neither from the PFG studied here was induced by the UMTS exposure. From obtained results we conclude that UMTS exposure at chosen conditions induced neither TP53 mutations nor TEL-AML1/MLL-AF4/MLL-AF9 preleukemic fusion genes as analyzed by the RSM or RT-qPCR technique, respectively, regardless the ability of UMTS exposure to induce DNA damage as measured by comet assay.

We found decreased yield of RNA per cell upon exposure to UMTS RF. To the best of our knowledge, this is the first report indicating that non-thermal RF exposure from mobile phone can affect a bulk RNA expression. Previous studies focused on analyzing expression of selected genes and transcriptome profiles (Belyaev et al., 2006; Nittby et al., 2008; Fragopoulou et al., 2018), and more recently on miRNA, which play key role in proliferation, differentiation, and apoptosis by suppressing specific target genes (Dasdag et al., 2015a, 2015b; Dasdag et al., 2019). Expression of multiple genes was shown to be either induced or suppressed by RF in these studies. Interestingly, expression of several miRNA was shown to be inhibited. In particular, Dasdag et al. found that long-term exposure of rats to RF at 2.4 GHz inhibited expression of some of the miRNAs such as miR-106b-5p and miR-107 (Dasdag et al., 2015a, 2015b). However, no study has so far provided the yield of RNA per cell to be compared with our results.

As far as non-thermal RF effects were shown to be accumulated during chronic exposures (Belyaev, 2017), further studies with prolonged exposures to different signals of mobile communication are warranted. These should include systems biology studies both *in vitro* and *in vivo*. More specifically, effects in critical biological processes, such as cell cycle, DNA replication and repair, RNA and protein expression, cell death, cell signaling, nervous system development and function, immune system response and carcinogenesis should be studied (Fragopoulou et al., 2018).

5. Conclusion

We found relatively small but statistically significant induction of DNA damage in dependence on UMTS frequency channel with maximal effect at 1977 MHz through alkaline comet assay. We concluded that UMTS RF exposure at the 1923 and 1977 MHz frequency did not induce ROS, apoptosis, selected TP53 mutations and PFG, but inhibited a bulk RNA expression. Our data support a notion that each specific signal used in mobile communication should be

tested in specially designed experiments to rule out that prolonged exposure to RF of mobile communication would affect human population and biota.

Statement from authors

All of the authors have read and approved the paper and it has not been published previously nor is it being considered by any other peer-reviewed journal.

Author contributions

I.B. and S.G. conceived the experiments; S.G., P.K., M.D., M.S., L.J., E.M., conducted the experiments; S.G., P.K., M.D., M.S., I.B. analyzed the results; S.G and I.B. wrote the manuscript.

Declaration of competing interest

S.G., P.K., M.D., M.S., L.J., E.M., report no conflict of interest. IB provided expert opinions in the Cell Phone Litigation on link between microwave radiation from mobile phones/base stations and human health.

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Appendix A. Supplementary data

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