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Effect of electromagnetic field exposure on the transcription of repetitive DNA elements in human cells

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ABSTRACT

Repetitive DNA (RE-DNA) was long thought to be silent and inert; only recent research has shown that it can be transcribed and that transcription alteration can be induced by environmental stress conditions, causing human pathological effects. The aim of this study was to determine whether exposure to radiofrequency electromagnetic fields (RF-EMF) could affect the transcription of RE-DNA. To this purpose, three different human cell lines (HeLa, BE(2)C and SH-SY5Y) were exposed to 900 MHz GSM-modulated RF-EMF at specific absorption rate of 1 W/kg or to sham. After exposure, mRNA levels of RE-DNA were evaluated through quantitative real-time PCR. The following RE-DNA types were investigated: Long Interspersed nucleotide Element 1, DNA alpha satellite and Human Endogenous Retroviruses-like sequences. When comparing cells exposed to RF-EMF versus control samples, different results were found for the three cell lines evaluated, indicating that RF-EMF exposure can significantly affect RE-DNA transcription and that the effects strongly depend on the cellular context and the tissue type. Further studies are needed to elucidate which molecular mechanisms could be involved.

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Mobile phone radiation; alpha satellite DNA; HERV; LINE; retrotransposition

Introduction

In our everyday life environment, radiofrequency electromagnetic fields (RF-EMF) are ubiquitous due to the worldwide use of mobile phones. Over the last years, a growing concern has aroused about public health effects since in 2013 the International Agency for Research on Cancer classified RF-EMF as a possible carcinogen for humans (risk group 2B) (IARC 2013). Many studies have been carried out to evaluate the possible biological effects considering both in vivo systems, such as normal and transgenic animals, and in vitro models, such as cell lines and primary cells. Various biological endpoints have been analyzed, including genotoxicity, cell proliferation and apoptosis, signaling transduction pathways, gene expression, oxidative stress indicators (Herrala et al. 2018; Liu et al. 2015; Manna and Ghosh 2016; Tsoy et al. 2019; Zong et al. 2019). Some studies have detected significant biological effects induced by RF-EMF exposure (Alkis et al. 2019; Hancı et al. 2018; Salford et al. 2003), others did not (Gläser et al. 2016; Imai et al. 2011; Loughran et al. 2013; O'Connor et al. 2010). Recent review articles have summarized the current state of the knowledge (Simkó et al. 2016; Vijayalaxmi and Prihoda 2019) and it could be concluded that although various biological effects from RF-EMF exposure have been actually observed, their relevance to human health is still uncertain. On the other hand, also possible beneficial effects have been reported, including adaptive response: cells that were pre-exposed to RF-EMF became resistant to the damage induced by subsequent exposure to a genotoxic agent (reviewed by Vijayalaxmi et al. 2014).

Several studies dealt with gene expression, evaluated also by transcriptomics studies (McNamee et al. 2016; Sakurai et al. 2011), and some of them found that genes involved in stress response could be affected by RF-EMF exposure (Misa-Agustiño et al. 2015; Trivino Pardo et al. 2012). Recently it has been reported that 900 MHz RF-EMF exposure can alter some of the miRNA (Dasdag et al. 2015). None of these studies has yet considered the transcription of repetitive DNA (RE-DNA) elements, which constitute about one half of the human genome. RE-DNA was originally thought to be silent and inert so that it was first called "junk DNA". However, recent evidences clearly indicated that RE-DNA can be transcribed into repetitive noncoding RNA, which plays an important role in gene and genome regulation. Moreover, RE-DNA transcription levels have been shown to be altered by environmental stress exposure, influencing genome stability. This raised

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the question of whether RF-EMF exposure could affect the transcription of RE-DNA.

RE-DNA are usually classified into different classes, the two most abundant of which are transposable elements (TE) and non-coding tandem repeats. Retrotransposons are the only active TE in the human genome, since they retrotranspose through an RNA intermediate and are grouped in non-long terminal repeat (non-LTR) and long terminal repeat (LTR) elements.

In the following, we will consider three types of RE-DNA which have been object of this study: (1) Long INterspersed nuclear Element-1 (LINE-1 or L1), which is a non-LTR element; (2) Human Endogenous Retroviruses-like (HERV) which is an LTR element; (3) DNA satellite (SAT) which is a non-coding tandem repeat element.

(1) L1s are autonomous transposable elements constitute approximately 17% of the human genome (IHGSC 2004). A full length L1 element is about 6 kb and consists of a 5′ untranslated region (5′UTR) containing a promoter with a bidirectional orientation, two open reading frames (ORF1 and ORF2), encoding, respectively, a 40-kDa nucleic acid binding protein and 150-kDa protein with endonuclease and reverse transcriptase activities, and 3′ untranslated region (3′ UTR) with polyadenylation site. Although the majority of human L1s are defective, about 100 elements have been estimated retrotransposition competent and are able to copy themselves and insert new copies of their DNA into other sites in the genome, causing genomic instability by insertional mutagenesis, transduction deletions, insertions, recombination inversions and translocations (Beck et al. 2011). Retrotransposition process consists of several steps: transcription and translation of L1 RNA, reverse transcription and integration of retrotranscripts in a new genomic site by a molecular mechanism termed target-site primed reverse transcription (TPRT). L1 activity can also affect the expression of nearby genes altering transcription factor-binding sites, promoters, splice sites (Beck et al. 2011). It is well known that L1 activity is tightly repressed in most somatic cell types, except for neuronal cells during neurogenesis (Coufal et al. 2009; Giorgi et al. 2018; Macia et al. 2017), and various suppressive mechanisms act at each step of the retrotransposition process to ensure genomic stability (reviewed by Goodier 2016). However, several environmental agents can interfere with these suppressive mechanisms. Indeed, alteration of L1 activity has been observed as a consequence of exposure to chemicals and physical agents (reviewed by Miousse et al. 2015), including extremely low-frequency magnetic field (Del Re et al. 2012; Giorgi et al. 2017). These evidences suggest that L1 activity could be used as biomarkers of exposure to environmental stressors and could be introduced into safety and risk assessment (Miousse et al. 2015).

(2) HERV sequences are endogenous retroviruses residual of ancestral infections, which have been integrated as proviruses into the host genome. They constitute approximately 8% of the human genome (IHGSC 2004). Although many cellular mechanisms restrict HERV activity, some members of the HERV, including HERV-H, are still transcriptionally active (Pérot et al. 2012). Moreover, HERV sequences can act as transcription start sites, transcription termination sites, splice donor/acceptor sites. So they can play a role in the long intergenic non-coding RNA, regulatory system, altering gene expression and interfering with genome functionality (Wang et al. 2014). An overexpression of HERV-H has been associated with several complex diseases with multifactorial etiology, including neurodevelopmental disease (Balestrieri et al. 2014; Cipriani et al. 2018) and it has been hypothesized that HERVs could represent the link between genetic and environmental risk factors.

(3) SAT sequences constitute about 15% of human genome. In particular, alpha satellite DNA (SATA), consisting of 171-bp monomeric repeat units, are present in all human chromosomes and enriched at all human centromere. The monomer arrays are chromosome specific but share a high homology in consensus sequence. Some monomers within the centromere contain a 17 bp CENP-B box, which is a binding site for the CENP-B protein, that is used for centromere assembly, so SATA are essential for centromere function during cell division. These sequences are transcribed into repetitive noncoding RNA and this transcription, tightly regulated, is needed for the accurate cell division (McNulty et al. 2018). Numerous evidences indicate that this transcriptional activity can be altered by various environmental chemical and physical agents (reviewed by Ferreira et al. 2015).

All these observations show that alteration of RE-DNA transcription can be caused by environmental agents and could contribute to disease onset. This research addresses the question of whether RF-EMF exposure can affect the transcription of RE-DNA, a topic which, as far as we known, has not yet been investigated. To this purpose, we evaluated the mRNA levels of L1, HERV-H and SATA elements in three different human cell lines exposed to 900 MHz GSM-modulated RF-EMF at specific absorption rate (SAR) of 1 W/kg and compared with those of the sham-exposed control. 900 MHz is an important frequency for mobile communication electromagnetic radiation, emitted from GSM mobile phones and from their base station antennas.

Materials and methods

Cell culture

HeLa cells, neuroblastoma BE(2)C cells, and neuroblastoma SH-SY5Y cells were kindly provided by Prof. Perini (University of Bologna, Italy). All cell lines were grown in DMEM medium (EuroClone, Milano, Italy), containing 10% heat-inactivated fetal bovine serum (EuroClone), 100 UI/ml penicillin (Sigma, Ronkonkoma, NY, USA) and 100 µg/ml streptomycin (Sigma), under a 5% CO2 humified atmosphere at 37°C.

In each assay, cells were plated in four-well plates (15 mm diameter; 1 ml/well; culture area 1.9 cm^2) (Thermo Scientific Nunc, A/S Roskilde, Denmark) at a density of 80000 cells/well and allowed to adhere overnight, then the plates were randomly assigned to each Transverse Electro Magnetic (TEM) chamber and exposed to 900 MHz GSM-modulated RF-EMF at specific absorption rate (SAR) of 1 W/kg or to sham.

Nucleic acid extraction and reverse transcription

Total RNA was extracted using RNaeasy (Qiagen, Milano, Italy), according to the manufacturer's recommendations, treated with Rnase-free Dnase (Qiagen) and quantified by Nanodrop fluorometry (Thermo scientific, Wilmington, Del., USA). RNA (1 µg) was reverse-transcribed by the Omniscript reverse transcription (RT) Kit (Qiagen) with random primers (Sigma).

Quantitative real-time polymerase chain reaction (real-time PCR)

Real-time polymerase chain reaction (PCR) was carried out by using the iQ SYBR green Supermix (Bio-Rad Laboratories, Segrate, MI, Italy) and oligonucleotides specific for the gene of interest (Table 1). Primers to amplify L1 ORF2, HERV-H, and SATA elements were designed by Coufal et al. (2009). After amplification, the specificity of the reaction was assessed by analyzing melting profile. mRNA levels were evaluated by the comparative threshold cycle number (Ct) method using the following formula that is described in detail in Pfaffl et al. (2002):

$$Ratio = \left(E_{target}\right)^{\Delta Ct\ target(control-treated)} / \left(E_{ref}\right)^{\Delta Ct\ ref(control-treated)}$$

Briefly, the relative expression ratio of a target gene is based on its real-time PCR efficiencies (E) and the threshold cycle (Ct) difference between sham-exposed samples (control) and RF-EMF exposed samples (treated), normalized against the mRNA levels of housekeeping gene. For each real-time experiment, both the D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene and 18S ribosomal RNA (18S rRNA) were used as a housekeeping reference genes.

Table 1. List of forward and reverse sequences of primers used.

Gene	Forward primer	Reverse primer
GAPDH	GCTCTCTGCTCCTCCTGTTC	GACTCCGACCTTCACCTTCC
18S rRNA	CTAAGGGCATCACAGACC	TCCGATAACGAACGAGAC
L1-ORF2	TGCGGAGAAATAGGAACACTTTT	TGAGGAATCGCCACACTGACT
HERV-H	AATGGCCCCACCCCTATCT	GCGGGCTGAGTCCGAAA
SATA	GGTCAATGGCAGAAAAGGAAAT	CGCAGTTTGTGGGAATGATTC

Exposure system

Prof. Laura Calzà (University of Bologna) kindly loaned us the exposure system she previously used (Del Vecchio et al. 2009a, 2009b). The exposure system and the dosimetry were described in great detail in one of these papers (Del Vecchio et al. 2009b). Briefly, the system is composed of two twin "Transverse Electro Magnetic (TEM) cells" with two independent power supply chains (two GSM signal generators and two EMF amplifiers) to obtain sham and exposed samples at the same time and it was positioned inside a Heraeus incubator (B-5060, Heraeus, Hanau, Germany) (37°C, 5% CO2). Each TEM cell has a squared cross-section (14 cm wide), and can contain 6 four-multiwell plates (66 mm x 66 mm external size). The RF-EMF (900 MHz; GSM basic with pulsed modulation at 217 Hz) is uniformly absorbed in the two wells of the two symmetric plates closer to the inner copper septum of the TEM cell resulting in a SAR of 1 W/Kg. Therefore, only the septum-adjacent wells were used in the experiments according to Del Vecchio et al. (2009a, 2009b).

The system was controlled by LabVIEW 6.1 software (National Instruments, Austin, TX). Experiments were conducted in blind modality where the researchers who performed biological assays did not know which of the two TEM chambers was active, and which acted as sham.

In each assay, cells were plated and allowed to adhere overnight, then were exposed to 900 MHz GSM-modulated RF-EMF at SAR of 1 W/kg or to sham. After 48 h exposure, RNA was extracted from each sample, retrotranscribed and cDNA were amplified and quantified using quantitative Real-time PCR by specific primers (Table 1).

Statistical analyses

Relative Expression Software Tool (REST) 2009 v.1.3.5 software was used. REST 2009 was created by Corbett Research Pty Ltd (QIAGEN Group) and Dr. Michael W. Pfaffl (Technical University Munich), to statistically

test qPCR data. It is based on the mathematical model described by Pfaffl et al. (2002) and allows a comparison of target genes with reference genes in two experimental groups, testing the differences between groups for significance by pairwise fixed reallocation randomization test (Pfaffl et al. 2002). Differences were considered significant starting from $p < .05$.

Results

The aim of the present research was to verify whether RF exposure could affect the transcription of RE elements such as L1, HERV-H and SATA sequences. This research has been carried out using, as a model, the epithelial human HeLa cell line, which has been used in many studies dealing with biological effects of RF-EMF exposure (Simkó et al. 2016), BE(2)C and on SH-SY5Y cell lines, which are neuronal-like cells often employed as in vitro models of neuronal function, differentiation and pathology (Biedler et al. 1978: Xicoy et al. 2017).

In all the figures, data are reported as relative expression (exposed samples/sham-exposed samples). In each test, both a single-copy reference gene (GAPDH) and a multi-copy reference gene (18S Rib) were used, and the related data are reported on the right and left side of the graphs, respectively. Figure 1 shows data for L1 transcription in the three cell lines. RF-EMF exposed HeLa cells exhibited a significantly lower mRNA level of L1 than sham-exposed control cells ($p < .02$ using GAPDH as reference gene; $p < .03$ using 18S rRNA as reference gene). No significant difference between exposed and sham-exposed was observed for BE(2)C cells. Finally, for SH-SY5Y cells, RF-EMF exposed ones showed a significantly higher L1 mRNA level than sham-exposed ones ($p < .03$ using GAPDH as reference gene; $p < .04$ using 18S rRNA as reference gene). To assess whether the transcription of the two reference gene could be affected by RF-EMF exposure, in each experiment mRNA levels of 18S rRNA were quantified using GAPDH as reference gene in both exposed and control samples. No difference was found in all the three cell lines (Figure 2). Therefore, the transcription of the reference genes was not affected by RF-EMF exposure.

Figure 3 shows data for HERV-H transcription. In Hela cells, HERV-H mRNA level resulted significantly lower in RF-EMF exposed cells than in sham-exposed ones ($p < .03$). For SH-SY5Y cells, the same comparison showed significantly higher HERV-H mRNA levels ($p <$ 0,02 using GAPDH as reference gene; $p < .01$ using 18S rRNA as reference gene). BE(2)C cells did not show any significant difference between exposed and sham-exposed cells. Therefore, also in this case, different results were obtained in the three cell lines.

Similar results were obtained analyzing SATA mRNA levels (Figure 4) which resulted significantly decreased in Hela cells exposed to RF-EMF as compared to sham control (<0.02 using GAPDH as reference gene; $p < .01$ using 18S rRNA as reference gene), significantly increased in SH-SY5Y cells ($p < .03$ using GAPDH as reference gene; $p < .01$ using 18S rRNA as reference gene), unchanged in BE(2)C cells.

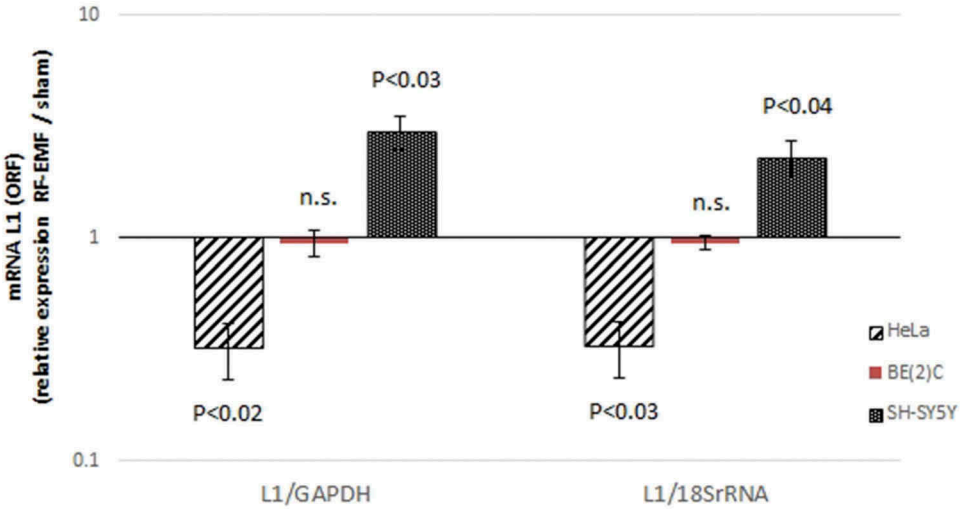


Figure 1. Effects of RF-EMF exposure on L1 mRNA levels in HeLa, BE(2)C and SH-SY5Y cells. Data are expressed as relative expression (RF-EMF exposed cells over sham-exposed cells) and reported on the logarithmic scale. The three histograms on the right side of the graph show data obtained using GAPDH as reference gene. The three histograms on the left side of the graph show data obtained using 18S rRNA as reference gene. Error bars represent SEM of the values obtained from five independent experiment. REST 2009 v.1.3.5 software was applied for statistical analysis, statistically significant p values are reported on the graph.

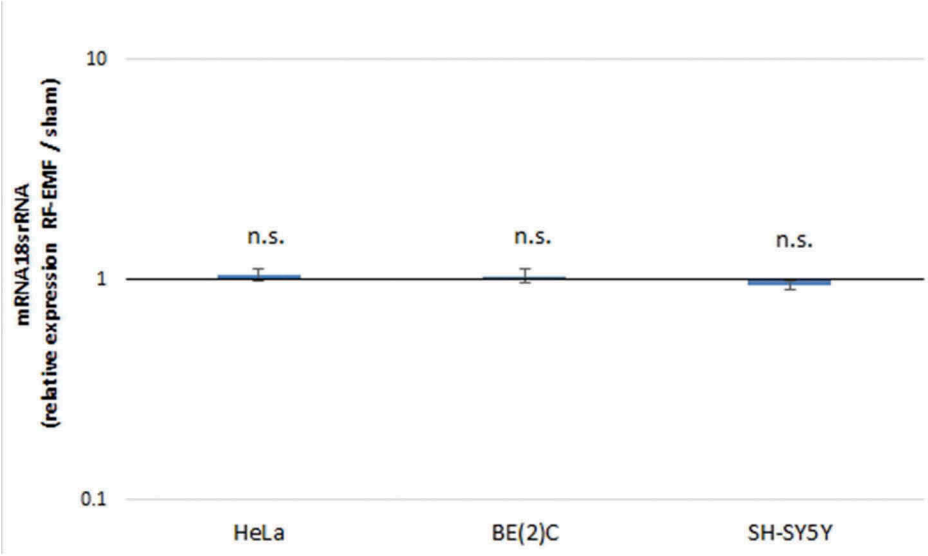


Figure 2. Expression of 18S rRNA in HeLa, BE(2)C and SH-SY5Y cells exposed to RF-EMF or to sham. In all the experiments, GAPDH was used as reference gene. Data are expressed as relative expression (RF-EMF exposed cells over sham-exposed cells) and reported on the logarithmic scale. Error bars represent SEM of the values obtained from five independent experiments. REST 2009 v.1.3.5 software was applied for statistical analysis, no significant *p* values were found.

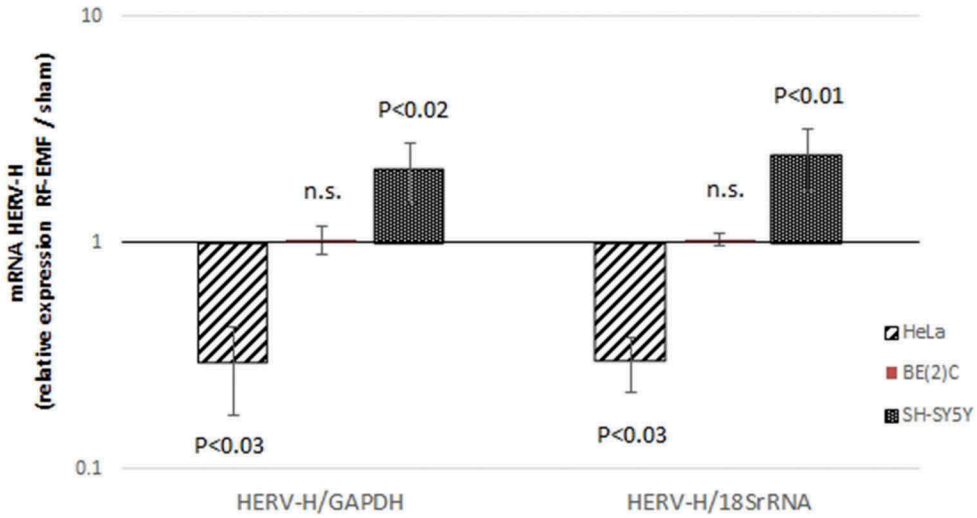


Figure 3. Effects of RF-EMF exposure on HERV-H mRNA levels in HeLa, BE(2)C and SH-SY5Y cells. Data are expressed as relative expression (RF-EMF exposed cells over sham-exposed cells) and reported on the logarithmic scale. The three histograms on the right side of the graph show data obtained using GAPDH as reference gene. The three histograms on the left side of the graph show data obtained using 18S rRNA as reference gene. Error bars represent SEM of the values obtained from five independent experiments. REST 2009 v.1.3.5 software was applied for statistical analysis, statistically significant *p* values are reported on the graph.

Discussion

Many in vitro studies have been carried out to assess whether RF-EMF exposure could affect gene expression (Dasdag et al. 2015; McNamee et al. 2016; Misa-Agustiño et al. 2015; Remondini et al. 2006; Sakurai et al. 2011; Trivino Pardo et al. 2012) but RE-DNA have never been analyzed since it has long been considered junk DNA. Only recent research has shown that RE-DNA can be transcribed and can play an important role in the cell functionality, critically influencing gene and genome regulation. In the present research, we evaluated mRNA levels of three different types of RE-DNA (L1, HERV-H, SATA) for three different cell lines exposed to RF-EMF or to sham. Our results show that RF-EMF exposure can affect the transcription of all the three RE-DNA analyzed, whereas it does not alter

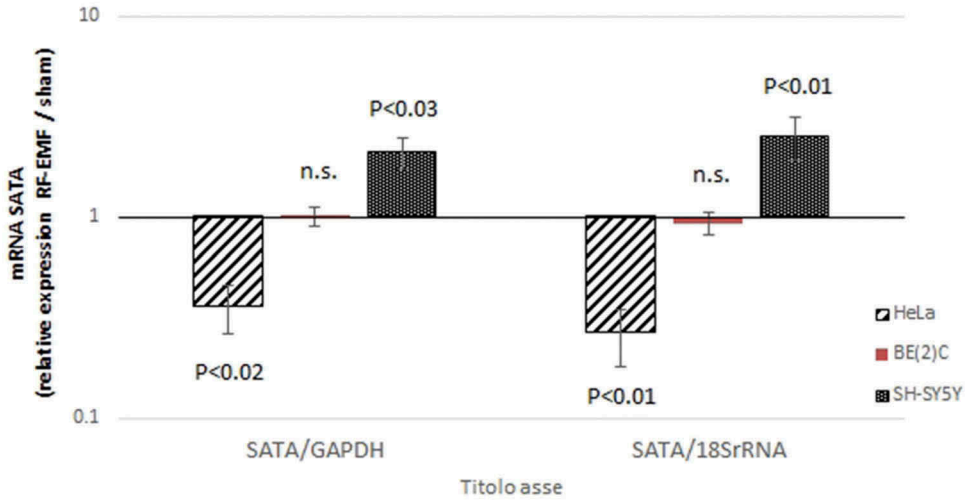


Figure 4. Effects of RF-EMF exposure on SATA mRNA levels in HeLa, BE(2)C and SH-SY5Y cells. Data are expressed as relative expression (RF-EMF exposed cells over sham-exposed cells) and reported on the logarithmic scale. The three histograms on the right side of the graph show data obtained using GAPDH as reference gene. The three histograms on the left side of the graph show data obtained using 18S rRNA as reference gene. Error bars represent SEM of the values obtained from five independent experiments. REST 2009 v.1.3.5 software was applied for statistical analysis, statistically significant *p* values are reported on the graph.

mRNA levels of the constitutive genes (GAPDH and 18S rRNA) indicating that it cannot affect constitutive transcriptional regulation.

Several studies indicate that the regulation of RE-DNA depends mainly on epigenetic mechanisms. L1 transcription is repressed by methylation of CpG in the L1 promoter, histone modifications, small RNA-mediated mechanisms (Idica et al. 2017). HERV expression depends on CpG methylation and histone deacetylation which affects the accessibility of transcription factors to the promoter (Maksakova et al. 2008). SATA transcription is mainly regulated by histone modification (McNulty and Sullivan 2018). Since epigenetic mechanisms are environmentally responsive, it is not surprising that RE-DNA expression can be affected by environmental condition. The alteration of the expression of RE-DNA that we observed could be explained by epigenetic changes triggered by RF-EMF. This topic is still little studied, but recent researches have reported that RF-EMF exposure can affect epigenetic mechanisms (Dasdag et al. 2015).

Our data indicate that the effect of RF-EMF exposure on the transcription of RE-DNA elements, probably mediated by epigenetic alterations, depends on the cellular system considered. RF-EMF exposure induced a downregulation and an upregulation, respectively, in epithelial-like cells (Hela) and in neuronal-like cells (SHSY5Y). However, no effect was observed in neuronal-like BE(2)C cells, indicating that the genetic background could be more important than the tissue type. As an example, we can cite the fact that BE(2)C and SHSY5Y cells differ from each other for p53 pathway status. p53 gene, often referred as the 'guardian of the genome', codifies for a protein that regulates the expression of hundreds of genes of multiple pathways involved in cellular response to various types of stress (Hafner et al. 2019). BE(2)C cells have been shown to be p53 mutant and resistant to a variety of chemotherapeutic agents, whereas SHSY5Y have been shown to be p53 wild-type (Carr et al. 2006). This difference could influence the cell epigenetic mechanisms. Evidence that genetic background is an important factor, which directly affect the L1 activity in response to environmental stress, was recently reported by Miousse et al. (2017). They found differences in the expression of L1 elements in response to ionizing radiation between the two mouse strains used (relatively radioresistant C57BL/6J and radiosensitive CBA/J mice).

Here we reported mRNA level changes but we do not know whether this biological effect is enduring or transient, what these changes mean and whether they can affect cell functionality. The increase of RE-DNA mRNA induced by RF-EMF exposure could result in cellular dysfunction. Indeed, L1 transcription increase is observed in cancer cells and has been considered to play a role in tumorigenesis and/or tumour progression (reviewed by Burns 2017). Increased transcriptional activity of HERV-H sequences has been found in different types of cancer and in several neurological and

psychiatric diseases (Balestrieri et al. 2014; Cipriani et al. 2018) suggesting that HERV-H transcription could take part in many multifactorial complex diseases. SAT DNA aberrant transcription was found in several tumor types suggesting that altered transcription levels are detrimental and may play a role in tumorigenesis or cancer progression (Ferreira et al. 2015), causing a chromosome missegregation in anaphase and aneuploidy.

Therefore, the increase of the mRNA levels of L1, HERV-H, and SATA, observed in exposed SHSY5Y cells, could cause both genomic instability and alteration of the expression of other genes, suggesting that RF-EMF exposure would be detrimental to cell function. On the other hand, we also observed that RF-EMF exposure could induce decrease of RE-DNA mRNA levels, as happened in Hela cells, resulting in an effect against the onset of genomic instability.

In conclusion, our data suggest that RF-EMF exposure affects RE-DNA transcription depending on the cellular context and encourage to carry out further studies to analyze this phenomenon and to evaluate whether epigenetic effects are involved.

Disclosure statement

The authors declare that they have no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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