Original Article

Extremely Low Frequency Magnetic Fields Induce *mTOR* and *Hsa_Circ_100338* Expression Changes in Gastric Cancer and Normal Fibroblast Cell Lines

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Abstract

Objective: Extremely low-frequency magnetic field (ELF-MF) exposure, as a targeted tumor therapy, presents several potential advantages. In this research, we investigated effects of different ELF-MF intensities on cell viability and expression levels of the mammalian target of rapamycin (mTOR) and hsa_circ_100338 in the normal fibroblast (Hu02) and human gastric adenocarcinoma (AGS) cell lines.

Materials and Methods: In this experimental study, cell lines of AGS and Hu02, were cultured under the exposure of ELF-MF with magnetic flux densities (MFDs) of 0.25, 0.5, 1 and 2 millitesla (mT) for 18 hours. The 3-(4, 5-dimethylthiazoyl-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to evaluate the cell viability. Relative expression of mTOR and hsa_circ_100338 RNAs was estimated by quantitative real-time polymerase chain reaction (qRT-PCR) technique.

Results: Viability of the normal cells was significantly increased at MFDs of 0.5, 1 and 2 mT, while viability of the tumor cells was significantly decreased at MFD of 0.25 and increased at MFD of 2 mT. Expression level of mTOR was significantly increased at the all applied MFDs in the normal cells, while it was significantly decreased at MFDs of 0.25 and 0.5mT in the tumor cells. MFDs of 1 and 2 mT in tumor cells inversely led to the increase in mTOR expression. hsa_circ_100338 was downregulated in MFD of 0.25 mT and then it was increased parallel to the increase of MFD in the normal and tumor cells.

Conclusion: Results of the present study indicated that ELF-MF at MFDs of 0.25 and 0.5 mT can lead to decrease in the both mTOR and hsa_circ_100338 expression levels. Given the role of mTOR in cell growth, proliferation and differentiation, in addition to the potential role of hsa_circ_100338 in metastasis, expression inhibition of these two genes could be a therapeutic target in cancer treatment.

Keywords: Circulating MicroRNA, Gastric Cancer, Gene Expression, Magnetic Field, mTOR Protein

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Introduction

Today, due to the increasing presence of electromagnetic fields in daily life, its effects on health have received much attention. It has been found that magnetic fields can affect living systems, but their beneficial or harmful effects have not been fully investigated (1). Association of electromagnetic fields with risk of various cancers, including postmenopausal breast cancer (2), childhood leukemia (3) and lung cancer (4) has been demonstrated by some studies. It has been found that electromagnetic fields affects a variety of physiological processes (5), while such effects depend on extremely low-frequency magnetic field (ELF-MF) frequency and duration (6).

Plasma membrane structure, intracellular signal transduction pathways and gene transcription could be changed following the exposure of electromagnetic fields (7, 8).

Applying the electromagnetic field as a non-invasive technique was used in the oncology field to treat cancer cells. In EMF therapy micro-currents are exposed locally to the specific tissue or the entire body. ELF-MF is used for the treatment of several diseases like Parkinson disease (9). EMF therapy, in the wave range of 0-300 Hz can help reduce dosage, harmful side-effects of chemotherapy and radiotherapy, further to enhancing prognosis of the disease (10).

During the last few years, the mammalian target of rapamycin (*mTOR*) has received significant attention for its importance in treating cancer. *mTOR* plays an important role in cancer cell growth through affecting the activity of elF4F complex. This enzyme increases translation by phosphorylating two targets of ribosomal p70S6 kinase

(S6K1) and initiation factor 4E binding protein 1 (4E-BP1) (11). Stimulating expression of the membrane surface carriers is one of the most important roles of *mTOR* in the body, leading to the increase access to nutrients for cell growth and proliferation (12). Moreover, *mTOR* also enhances expression of hypoxia-inducible factor-1α (HIF-1α), which plays a key role in angiogenesis. Therefore, mutations in the upstream components of the *mTOR* pathway are implicated in cancer progression (13). Some studies indicated that *mTOR* dysfunction is applied as a therapeutic target in gastric cancer (GC). Additionally, increased *mTOR* activity is directly related to tumor progression and low patient survival (14).

Circular RNAs (circRNAs) are non-coding RNAs with stable structure and tissue-specific expression (15). Unlike other RNAs, circRNAs are resistant to degradation and they are highly ubiquitous in the transcriptome of eukaryotes (16). Some studies have shown that circRNAs can act as microRNA (miRNA) sponges, regulators of splicing and transcription, as well as altering gene expression (15). Changes in circRNA expression could induce abnormal cellular functions and various diseases including cardiovascular diseases and various cancers (17, 18). A recent study showed the clinical significance of *hsa_circRNA_100338* in hepatocellular carcinoma (HCC) and suggested that this circRNA could be a potential biomarker for HCC. The report also revealed that *hsa_circRNA_100338* could act as a sponge for miR-141-3p and increase potential of the liver cancer cells to metastases (19). Although some studies were done to provide *hsa_circRNA_100338* as a biomarker of cancer cells, there is no evidence to assay the expression level of *hsa_circRNA_100338* on GC cells at different electromagnetic flux densities.

The aim of this study was to investigate effect of magnetic flux densities (MFDs) of 0.25, 0.5, 1 and 2 millitesla (mT) on the expression level of *mTOR* and *hsa_circRNA_100338* in a human gastric cancer cell line (AGS) and human normal fibroblast (Hu02) cells after 18 hours.

Materials and Methods

In this experimental research, expression levels of *mTOR* and *hsa_circ_100338* were determined in AGS cell line, as tumor cell and Hu02 cell line, as normal cells, following the exposure to MFDs of 0.25, 0.5, 1 and 2 mT in comparison to the unexposed cells.

This study was approved by the Ethics Committee of Bushehr University of Medical Sciences (IR.BPUMS.REC.1339.180).

Cell culture

Human gastric adenocarcinoma (AGS) and human normal fibroblast (Hu02) cell lines were purchased from the National Genetic Resources Center of Iran, and then according to their instructions, the cells were seeded monolayer in the 25 ml flasks. AGS cells were cultured in Ham's F12 (Gibco, USA) medium as described in the previous study (20). The cells were exposed to ELF-MF with MFDs of 0.25, 0.5, 1 and 2 mT continuously for 18 hours.

Exposure system

Electromagnetic fields were produced by the system consisted of a solenoid cylinder of diameter 12 cm, length 30 cm and 1200 turns (21). To produce various electromagnetic intensities with a frequency of 50 Hz, an alternative current power supply (TDGC2, 220v, 50-60 Hz; Delta International Electric, China) was applied. The electromagnetic field generated inside the solenoid is uniform and in the direction of the cylindrical axis. The solenoid cylinder was placed horizontally at the center of the CO_2 incubator and flasks of cells were placed in the middle of the cylinder. The sham groups were incubated at the same condition inside the solenoid cylinder, while the circuit was interrupted. A digital teslameter with a 3-D sensor (Holaday, USA) was used to estimate the electromagnetic flux density at the middle of the solenoid cylinder.

MTT assay

The cell viability was determined by the 3-(4, 5-dimethylthiazoyl-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. For this purpose, the medium was removed from the cultured cells in the 96 well plates. Then, the MTT solution was added to each well after which the plate was incubated for 4 hours at 37°C with 5% CO_2. After removing supernatant, 100 µl Dimethylsulfoxide (DMSO) was added to each well and shaken well to dissolve all formazan crystals. Finally, the absorption was measured at 570 nm by a microplate reader (Synegry HT, USA).

RNA extraction, cDNA synthesis and real-time-polymerase chain reaction

To measure expression changes of *mTOR* and *hsa_circRNA_100338*, real-time PCR (qRT-PCR) was used. Briefly, total RNA was extracted by using TRIzol reagent (TRI Sigma-Aldrich, Germany) according to the manufacturer's instruction. Takara RNA PCR Kit was used to synthesize cDNA. Quantitative real-time PCR (qRT-PCR) was performed by Bioneer Exicycler™ 96 Detection System. Detail of the PCR steps have been described in our previous studies (20, 22). The primer sequences are listed in Table 1.

Delta threshold cycle value (ΔCt) was measured for each sample to determine the relative expression of genes to the housekeeping gene and then the delta delta threshold cycle value (ΔΔCt) which is the difference between ΔCt of the exposed tumor or normal cells and control group was determined. Fold changes of gene and miRs expression was calculated by $2^{-\Delta\Delta Ct}$ formula.

Table 1: The sequences of *GAPDH*, *mTOR*, and *hsa _circ_100338* primers used in the current study

Genes	Primer sequences (5′-3′)
hsa_circ_100338	F: AAAAGCAAGCAGTGCCCATA
	R: GCTCGAATCAGGTCCACCA
mTOR	F: AGCATCGGATGCTTAGGAGTGG
	R: CAGCCAGTCATCTTTGGAGACC
GAPDH	F: GCACCGTCAAGGCTGAGAAC
	R: GGATCTCGCTCCTGGAAGATG

Statistical analysis

SPSS software version 25 was used to analysis the data. The results were shown as mean ± standard deviation (mean ± SD). To compare significant differences among the groups, one-way and two-way analyses of variance and Tukey Post hoc test were used. The level of probability for the significant differences among the groups was considered as P<0.05.

Results

Cell viability measurement

ELF-MF with MFD of 0.25 mT did not affect cell viability of the normal cells while exposure to MFDs of 0.5, 1 and 2 mT lead to a significant increase in cell viability (P<0.0001).

Exposure of tumor cells to 0.25 mT MFD led to a significant decrease in cell viability, but the cell viability was increased in parallel with the increase of MFD and this increase was significant at MFD of 2 mT (Fig.1). Sidak analysis showed a significant decrease in tumor cells in comparison with normal cells in terms of viability at MFDs of 0.25 (P=0.02), 0.5 (P<0.0001), 1 (P<0.0001), and 2 mT (P<0.0001, Fig.1).

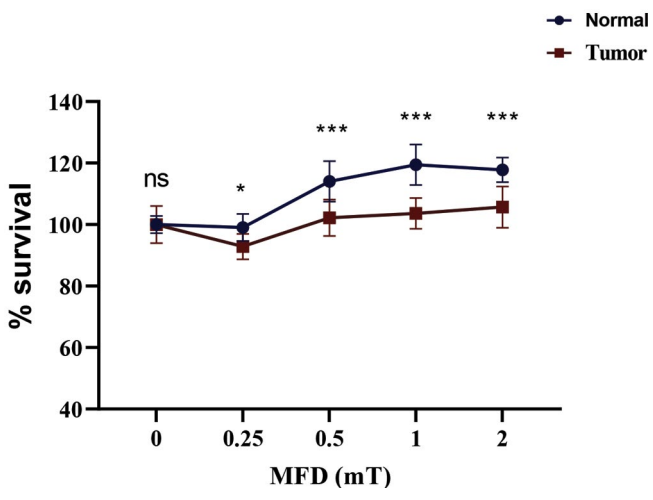

Fig.1: Mean viability percentage of the normal cells and tumor cells, following the exposure to continuous MFDs of 0.25, 0.5, 1 and 2 mT for 18 hours in comparison to the unexposed cells. *; P<0.05, ***; P<0.001, MFDs; Magnetic flux densities, and ns; Non significant.

Relative expression of *mTOR*

Expression level of the *mTOR* gene in the normal cells exposed to different intensities of ELF-MF was significantly higher than that of the unexposed normal cells (P<0.001). The highest and lowest expression level of this gene was observed at 2 mT and 0.5 mT respectively (Fig.2A). However, pattern of *mTOR* expression level in tumor cells was different from that in normal cells. By that means the expression level of the gene in exposed tumor cells at MFDs of 0.25 mT and 0.5 mT was downregulated and at MFDs of 1 and 2 mT was upregulated compared to unexposed tumor cells (Fig.2B). Expression level of the *mTOR* gene in the exposed and unexposed tumor cells, as well as the exposed and unexposed normal cells, was analyzed using the sidak test. As evident from the data shown in Figure 2C, the unexposed tumor cells showed no significant difference compared to unexposed normal cells concerning the relative expression of *mTOR* mRNA (P>0.05). However, relative expression of mTOR mRNA was significantly downregulated in the exposed tumor cells at MFDs of 0.25 (P<0.0001, 0.03 FC), 0.5 (P<0.0001, 0.05 FC), 1 (P<0.0001, 0.38 FC) and 2 (P=0.003, 0.68 FC) mT in comparison with the exposed normal cells (Fig.2C).

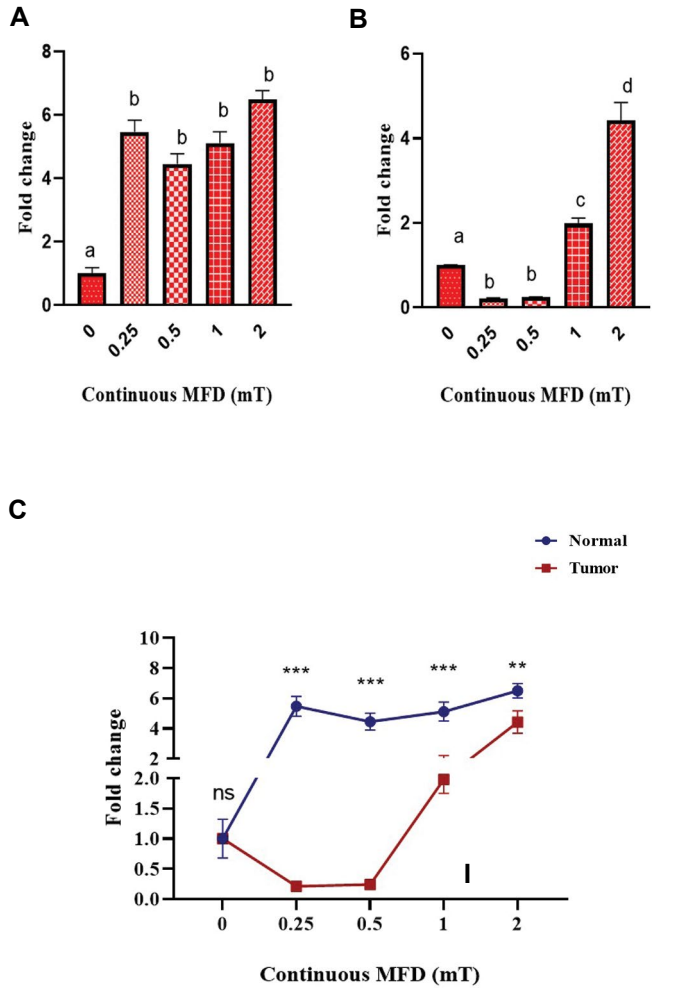

Fig.2: Comparison of *mTOR* expression levels in normal and tumor cells in different MFDs. mRNA expression level of *mTOR* in the **A.** Exposed normal and **B.** Gastric tumor cells at various MFDs. **C.** Comparison of the mRNA expression level of *mTOR* in the exposed, unexposed normal and tumor cells. Different letters on each column show statistically significant difference, and the same letters on each column has no difference. ***; P<0.001 and MFD; Magnetic flux densities.

Relative expression of *hsa_circ_100338*

Expression level of *hsa_circ_100338* in the exposed normal cells significantly down regulated at MFD of 0.25 mT (0.18 FC, P=0.0005) compared to the unexposed normal cells. At the other intensities, no significant change was observed (Fig.3A). However, relative expression of *mTOR* mRNA was significantly down-regulated in the exposed tumor cells at MFDs of 0.25 (0.002 FC, P<0.0001), 0.5 (0.013 FC, P<0.0001), 1 (0.19 FC, P=0.0016) and 2 mT (0.31 FC, P=0.017) in comparison with the unexposed tumor cells (Fig.3B). Sidak test was used to analyze expression level of the *hsa_circ _100338* in the all groups of tumor and normal cells. As shown in Figure 3C, the obtained data showed no significant difference between the unexposed tumor cells and unexposed normal cells. Concerning the expression level of *has-circ-0005986* (P>0.05). However, expression level of the *hsa _circ_100338* was significantly increased in the exposed tumor cells at MFDs of 0.25 (P<0.0001, 0.013 FC), 0.5 (0.024 FC, P<0.0001), 1 (0.16 FC, P<0.0001) and 2 (0.22 FC, P<0.0001) mT.

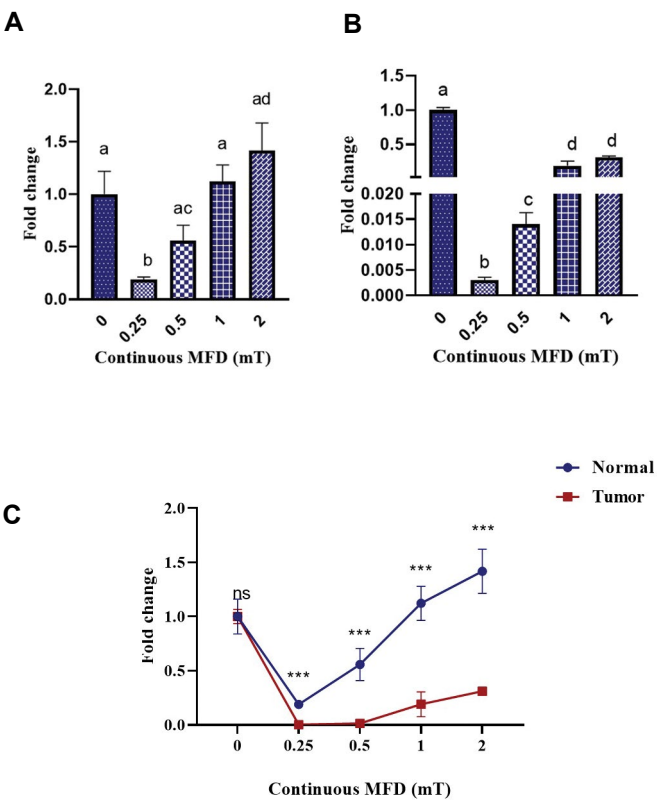

Fig.3: Comparison of *hsa_circ_100338* expression levels in normal and tumor cells in different MFDs. Relative expression level of *hsa_circ_100338* in the **A.** Tumor and **B.** Normal cells with different MFDs. **C.** Comparison of mRNA expression level of *hsa_circ_100338* in the exposed and unexposed normal cells with tumor cells. Different letters on each column show statistically significant differences, the columns having common letters show statistically no significant difference. ***; P<0.001, ns; Non significant, and MFDs; Magnetic flux densities.

Correlation analysis

A significant positive correlation (r=0.799, 95% CI=0.4866 to 0.9306, P=0.0003) was observed between expression level of the *hsa_circ_100338* and *(mTOR)* genes in the tumor cells exposed to extremely low frequency magnetic fields (ELF-MF). As shown in Figure 4, no significant correlation was observed between expression level of the *hsa_circ_100338* and mTOR genes in the normal cells exposed to ELF-MF (P=0.540).

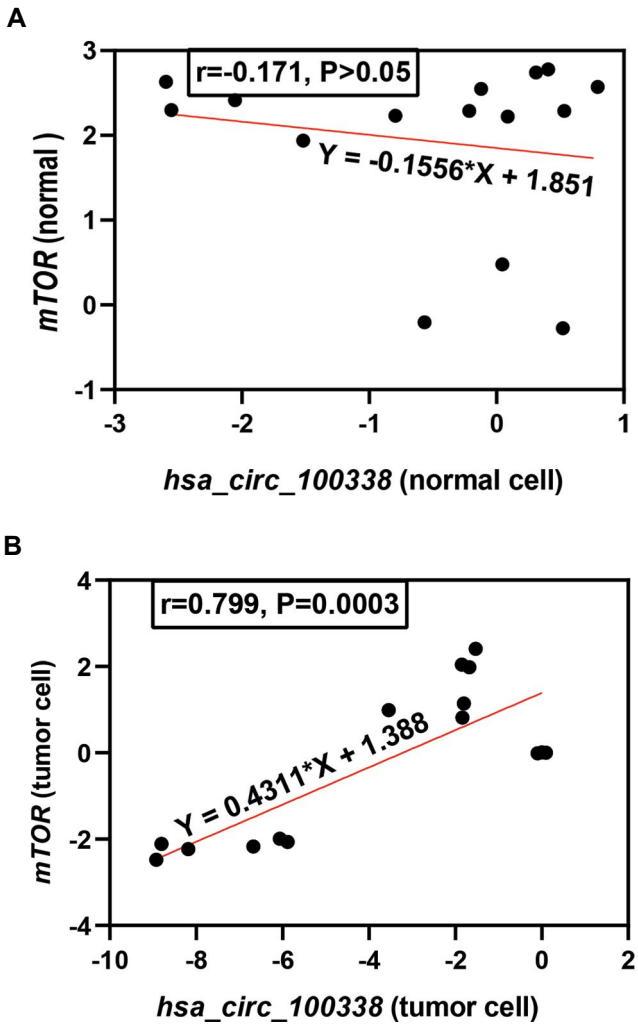

Fig.4: Correlation analysis between *mTOR* and *hsa_circ_100338* gene expressions in **A.** Normal cells and **B.** Gastric cancer cell lines exposed to extremely low frequency magnetic fields (ELF-MF).

Discussion

In the present study, effects of ELF-MF with MFDs of 0.25, 0.5, 1; and 2mT on AGS and Hu02 cell lines were investigated. Our finding indicated that ELF-MF can affect the cell viability and change the expression levels of *mTOR* and *hsa_circRNA_100338* in the both tumor and normal cells. Expression levels of *mTOR* were significantly increased at all applied MFDs in the normal cells while it was significantly decreased at MFDs of 0.25 and 0.5 mT in tumor cells. MFDs of 1 and 2 mT in tumor cells inversely lead to the increase in *mTOR* expression. *Hsa_circ_100338* was down-regulated in MFD of 0.25 mT and then was increased parallel to the increase in

MFD in normal and tumor cells.

Interaction of ELF-MF with living organisms could induce different biological effects that depend on the type, MFD, frequency and time of exposure (1, 23). There are some studies that indicated antitumor effect of EMFs (24). Release of intracellular proteases that alter the integrity of cancer cell membrane was reported in A549 human lung cancer cells exposed to 50 and 385 Hz electromagnetic fields (25). Decreased growth of breast cancer tumor cells under the influence of magnetic fields has been reported (26). These cytotoxic effects have been attributed to changes in plasma membrane properties, impaired Ca^{+2} penetration (27) and changes in cytoskeletal structure (28). In the present study, cytotoxic effects of the ELF-MF with MFD of 0.25 mT on gastric cancer cells were observed. However, some conflicting researches have been reported in this regard. Some researchers reported inducing effects of cell proliferation (29, 30) and the others demonstrated cytotoxic effects of EMFs (31, 32). Different results of magnetic fields in this study can be explained by EMFs window effects hypothesis. The window effect is a biological response that only occurs at certain criteria of the frequency, amplitude, intensity and time of exposure, while outside of these criteria, the biological system does not respond to EMFs (33). The intensities of 0.5-2 mT showed no decrease in cell viability of tumor cells but a relative increase in it, which indicate the anti-apoptotic effects of ELF-MF (34). It was shown that ELF-MF protected cancer cells from apoptosis due to heat shock (35). It was demonstrated that *PI3K/AKT/mTOR* pathway activation in GC could impair cell growth and proliferation as well as, metabolism and angiogenesis (14). In this study, it was shown that *mTOR* gene was overexpressed in the GC cell lines at 2 mT ELF-MF, but at MFDs of 0.25 and 0.5 mT, this gene was down-regulated in comparison with the unexposed GC cell lines. Phosphorylation of Ser2448 *mTOR* enzyme activates eukaryotic initiation factor 4E (elF4E) and ribosomal S6 kinase p70 (p70S6 kinase 1) and inactivates eIF4E inhibitor 4E-BO.1. In other words, overexpression of the *mTOR* gene upregulated expression level of cell growth and cell division mediators including cyclin D1, and down-regulated expression level of cell cycle progression regulators (36). Therefore, MFDs of 0.25 and 0.5 mT, which caused a sharp decrease in *mTOR* expression level of GC cell lines, can be considered as a treatment for gastric cancer.

Moreover, according to the results, expression level of *hsa_circ_100338* in AGS cell lines showed significant differences at different MFDs and this led to the downregulation of *hsa_circ_100338* expression level in an intensity-dependent manner. So that, the lowest expression level of *hsa_circ_100338* was seen at 0.25 mT ELF-MF. Nevertheless, by increasing, MFD of the electromagnetic field, its expression level was also increased. Few studies have been performed to determine the role of circRNAs in cancer. Our results suggested that *hsa_circ_100338* could be a good biomarker for diagnosis of gastric cancer. The biomarker role of *hsa_circ_100338* has been reported in HCC. It has been shown that *hsa_circ_100338* acted as a miR-141-3p sponge in HCC tissue (19). The other study revealed that circRNA-100338 was involved in regulation of angiogenesis and HCC metastasis (37). In the current study, therefore, *hsa_circ_100338* in AGS cells may also interact with miRNAs. Downregulation of *hsa_circ_100338* expression level was observed in AGS cells exposed to MFD of 0.25 mT.

Hsa_circ_100338 expression level had positive correlation with *mTOR* in the tumor cells following exposure of ELF-MF, whereas the normal cells treatment with ELF-MF showed no significant correlation. A study showed that *hsa_circ_100338* regulated protein kinase activity of *mTOR* in HCC patients and operated as a key regulator in promoting the *mTOR* signaling pathway. The study indicated that not only gene expression level of *hsa_circ_100338* was increased in HCC cells compared to the normal cells, but also knockdown of *hsa_circ_100338* gene affected cell proliferation ability (37).

ELF-EMFs can alter gene expression by several mechanisms, including alteration of cell membrane channels like calcium channels (38), increase in free radicals formation and stability (39), in addition to direct interaction with DNA (40). Overall, decrease of *mTOR* and *hsa_circ_100338* expressions as well as cell viability decrease in tumor cells, following the exposure to MFD of 0.25 mT in this study, indicated that this MFD had the most beneficial effects and it can be considered as an adjutant therapeutic method in cancer treatment.

Conclusion

Results of the present study indicated that ELF-MF at MFDs of 0.25 and 0.5 mT can down-regulated both *mTOR* and *hsa_circ_100338* expressions. Given the role of *mTOR* in cell growth, proliferation, differentiation and the potential role of *hsa_circ_100338* in metastasis, the expression inhibition of these two genes could be a therapeutic target in cancer treatment. However, more *in vitro* and *in vivo* researches are needed to find suitable MFD and exposure protocols to suppress and control cancer cell with fewer side-effects.

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Authors' Contributions

S.A.; Was the designer of the project and responsible for overall supervision. N.B.; Was involved in experimental studies and data analysis. F.M.; Carried out all experimental works, including primer design, cell culture,

qRT-PCR, and data analysis. M.E., A.D.; Were involved in experimental studies and drafting the manuscript. The authors have read and approved the final manuscript.

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