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RESEARCH ARTICLE

Thymidine decreases the DNA damage and apoptosis caused by tumor-treating fields in cancer cell lines

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Abstract

Background Tumor-treating fields (TTFields) is an emerging non-invasive cancer-treatment modality using alternating electric fields with low intensities and an intermediate range of frequency. TTFields affects an extensive range of charged and polarizable cellular factors known to be involved in cell division. However, it causes side-effects, such as DNA damage and apoptosis, in healthy cells.

Objective To investigate whether thymidine can have an effect on the DNA damage and apoptosis, we arrested the cell cycle of human glioblastoma cells (U373) at G1/S phase by using thymidine and then exposed these cells to TTFields.

Methods Cancer cell lines and normal cell (HaCaT) were arrested by thymidine double block method. Cells were seeded into the gap of between the insulated wires. The exposed in alternative electric fields at 120 kHz, 1.2 V/cm. They were counted the cell numbers and analyzed for cancer malignant such as colony formation, Annexin V/PI staining, γH2AX and RT-PCR.

Results The colony-forming ability and DNA damage of the control cells without thymidine treatment were significantly decreased, and the expression levels of BRCA1, PCNA, CDC25C, and MAD2 were distinctly increased. Interestingly, however, cells treated with thymidine did not change the colony formation, apoptosis, DNA damage, or gene expression pattern.

Conclusions These results demonstrated that thymidine can inhibit the TTFields-caused DNA damage and apoptosis, suggesting that combining TTFields and conventional treatments, such as chemotherapy, may enhance prognosis and decrease side effects compared with those of TTFields or conventional treatments alone.

Keywords Tumor-treating fields · cancer cell · DNA damage · Cell division · Thymidine

Introduction

Tumor treating fields (TTFields) is a non-invasive cancer treatment strategy involving alternating electric fields within the intermediate frequency range (100–300 kHz) at low-intensities (Kirson et al. 2004, 2007). In previous studies, TTFields has been shown to be effective in solid tumors, such as a brain tumor and non-small cell lung cancer, in vitro and in vivo (Jo et al. 2018; Kirson et al. 2004, 2007, 2009a, b). However, this treatment modality can cause DNA damage and apoptosis in healthy cells (Jo et al. 2018), because it interrupts the formation of the mitotic spindle apparatus and activation of the mitotic spindle checkpoint in all dividing cells. In addition, the anticancer effects of TTFields disturb a multitude of biological processes, including DNA repair, autophagy, cell migration, cellular permeability, and immunological responses (Kim et al. 2016, 2019; Kirson et al. 2007; Park et al. 2019). TTFields create a heterogeneous intracellular environment, which induces a

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di-electrophoretic movement of polar molecules toward the region of high field intensity, effectively preventing polymerization and other critical biochemical events (Gonzalez and Remcho 2005). Recent studies have provided insights into the molecular mechanisms underlying the anti-cancer effect of TTFields. In dividing cells, TTFields arrests the cell cycle at G/M phase and induces apoptosis (Giladi et al. 2015). These antimitotic effects of TTFields stand out in rapidly dividing cells, such as cancer cells, relative to healthy cells.

The anticancer effects of TTFields led to the first pilot study (EF-07) in glioblastoma patients in 2004, and eventually, to the development of TTFields as a common strategy for treating cancer in general. These pilot clinical trials and a randomized phase-III trial (EF-14) have shown significant increases in median overall survival in patients treated with a combination of TTFields and temozolomide chemotherapy by 4.9 months (Stupp et al. 2015, 2017). Following these results, TTFields was approved by the FDA in 2015 for the treatment of newly diagnosed glioblastoma multiform (GBM).

In this study, we investigated whether thymidine have an effect on the DNA damage and apoptosis in the human glioblastoma cells (U373) at G1/S phase by using thymidine and then exposed the cells to TTFields. Interestingly, we found that thymidine inhibited the TTFields-caused DNA damage and apoptosis, suggesting that combining TTFields and conventional treatments, such as chemotherapy, may enhance prognosis and decrease side effects compared with those of TTFields or conventional treatments alone in the future.

Materials and methods

Generation of electric fields (EFs)

EFs were generated using a pair of insulated wires (Seoil Electric Wire Co. Ltd; outer diameter, 0.4 mm; polyvinyl chloride insulation thickness, 0.17 mm; dielectric breakdown, 25 kV/mm) connected to a function generator (AFG-2112, Good Will Instrument Co., Ltd, Taiwan) and high-voltage amplifier (A303, A. A. Lab Systems Ltd, Israel) that generated sine-wave signals (0–800 V). The coiled wires were affixed to the bottom of each well at a distance of 1 cm from each other. The EF intensity in the medium was measured using two insulated wires with exposed tips, which were connected to an oscilloscope (GDS-2102 A, Good Will Instrument Co. Ltd, Taiwan) through a differential probe. The electric field intensities were expressed in peak voltage amplitude per centimeter (v/cm). The temperature within culture dishes was measured during the treatment since the application of electric fields can generate non-negligible heat. The fluctuation in temperature during the TTFields under our experimental conditions was found insignificant, and the maximum increase in temperature was ~0.3 ℃.

Cell culture in TTFields

Human U87 (1.0×10^5/well) and U373 (1.0×10^5/well) glioma cells and HaCaT keratinocytes (5.0×10^4/well) each were resuspended in 100 µl medium (DMEM, 10%FBS, 1% Penicillin/streptomycin), and were seeded between the wires on the bottom of each well of 6-well culture plates. The plates were then incubated in a humidified incubator at 37 °C in 5% CO_2/95 % air for 20 min for the cells to attach to the plates. Subsequently, 3 ml medium was added to each well, and the plates were returned to the incubator. Next day, electric fields were applied, and the plates were incubated for 72 h. Afterwards, the wells were washed with PBS, and the cells were detached via trypsinization and then counted using a hemocytometer. Cell-growth rate was defined as the ratio of the number of cells after EF treatment to the number of seeded cells, and relative cell-growth rate was defined as the ratio of the growth rate of EF-treated (experimental group) cells to the growth rate of untreated (control) cells.

G1/S cell-cycle synchronization via double thymidine block

For early–S-phase arrest, cells were cultured with 2 mM thymidine for 18 h. Subsequently, they were washed with PBS and incubated with fresh medium for 9 h. Then, the cells were cultured with 2 mM thymidine for 15 h more. At the end of the second thymidine block, the cells were washed with PBS and provided with fresh growth media. The thymidine-treated cells were exposed to TTFields in the thymidine-containing medium.

Analysis of apoptotic cells through annexin-V staining

At the end of TTFields, the cells were washed with ice-cold PBS. The cells were trypsinized, washed with ice-cold PBS, and resuspended in the 1X binding buffer of the Annexin V–FITC apoptosis detection kit (BioVision, Milpitas, CA, USA). The cell solution was mixed with Annexin V-FITC and 5 µl of propidium iodide via gentle vortexing, followed by 5 min of incubation in the dark at 22–25 °C. And then all the samples were analyzed on an Accuri C6 (BD Biosciences, Franklin Lakes, NJ, USA).

Immunofluorescence assay

Cells were fixed using 4 % formaldehyde solution in PBS at room temperature (RT) for 1 h. Afterward, the cells were washed with PBS twice. Then, they were permeabilized

using 0.1 % triton X-100 (Sigma, St.Louis, MO, USA) for 5 min at RT. The cells were blocked with 10 % normal donkey serum (Sigma) in PBS for at least 1 h at RT. Subsequently, they were incubated with anti-γH2AX antibody (1:500; Millipore) overnight at 4 ℃. Afterwards, the cells were incubated with secondary antibodies conjugated to Alexa Fluor 488 (1:1,000; Invitrogen). All the immunofluorescence images were obtained using a confocal microscope (Olympus, Japan).

Polymerase chain reaction (PCR)

Total cellular RNA was harvested using TRI reagent (Thermo Fisher, Waltham, MA, USA) and reverse-transcribed to cDNA by using M-MLV reverse transcriptase (Roche, Switzerland) and oligo dT primers, following the instructions of the manufacturers. Reverse transcription–quantitative PCR (RT-qPCR) was performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) with KAPA SYBR FAST ABI prism qPCR kit (KAPA Biosystems, Woburn, MA, USA).

Results

TTFields inhibits the growth of cancer cell lines but not the growth of healthy cells

We have previously shown that low-intensity and intermediate-frequency TTFields suppresses the growth of glioblastoma cells (Jeong et al. 2014; Jo et al. 2019). To examine whether cell-cycle–arrested cancer cells are responsive to TTFields, healthy or cancer human brain cells were exposed to TTFields under our well-established conditions.

First, to verify that TTFields inhibits cell growth, we cultured glioblastoma cells (U373), glioma cells (U87), and human adult keratinocytes (HaCaT) in the gap of the coiled-insulated wire. TTFields was performed with different intensities and a fixed frequency of 150 kHz for 72 h (Fig. 1). Afterwards, viable cells were counted using trypan blue. Relative cell number was defined as the ratio of the growth rate of the TTFields-treated cells to the growth rate of untreated cells.

The growth rate of the cells was declined as the electric-field intensity was gradually increased. At 1.2 V/cm, the growth rates of the cancer cell lines were 0.6, but the growth rate of HaCaT was > 0.8.

These results demonstrate that our TTFields system suppressed the growth of the cancer cell lines but not that of healthy keratinocytes.

The effects of TTFields are similar in both cell-cycle–arrested cancer cell lines and healthy cells

Our previous study has reported that TTFields increases the DNA replication stress and the number of double-strand breaks in cancer cells but not in healthy cells, which have relatively lower division rates than cancer cells.

To examine whether cell-cycle–arrested cancer cell lines showed the similar TTFields effects to those in healthy cells, we treated cancer cell line U373 with thymidine, thereby inducing their G1/S arrest (U373-thy). The U373 cells treated with or without thymidine were exposed to TTFields for 48 h, and the cell lines exposed to TTFields were seeded in 35 mm dish (5×10^2 viable cells) for colony formation assay. Our results showed that the cells exposed to TTFields had decreased colony forming abilities compared with that of the control cells, but the number of the colonies formed by U373-thy cells after TTFields was similar to that of control (Fig. 2). These results suggest that TTFields is not effective in the cell-cycle–arrested cancer cells.

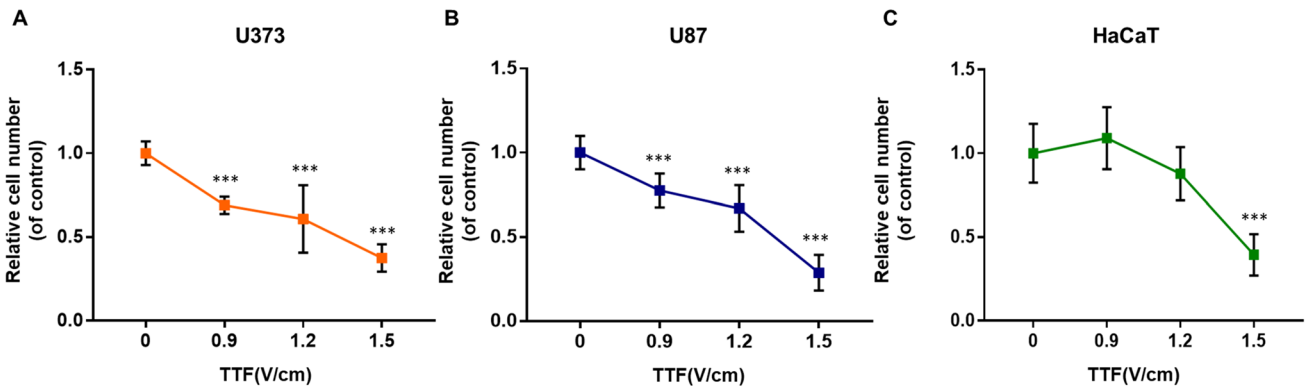

Fig. 1 The rate of cell growth after TTFields is dependent on the intensities and frequencies of the electric fields. *< 0.05, **< 0.005, ***< 0.0005

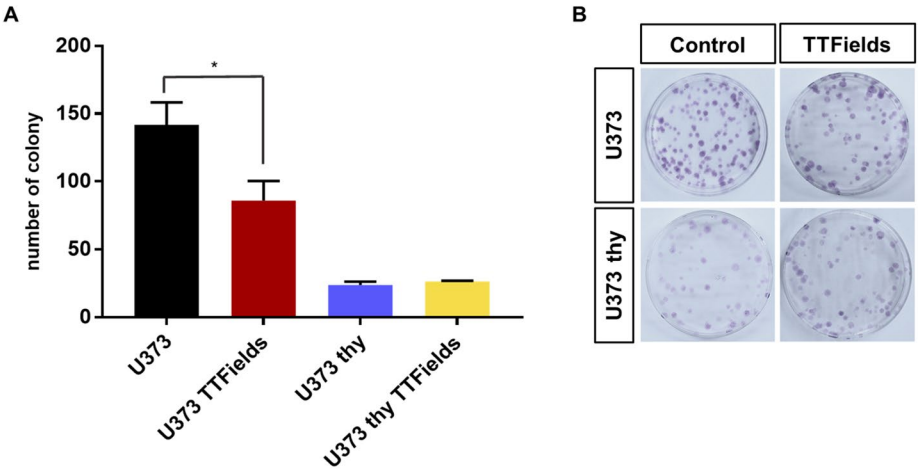

Fig. 2 Colony-forming ability after TTFields. **a** Number of colonies per well. *< 0.05, **< 0.005, ***< 0.0005. **b** Representative images of the colonies

TTFields does not trigger DNA damage or apoptosis when the cell cycle is arrested

Next, we examined whether TTFields was effective on thymidine-arrested cancer cell lines. U373 cancer cell line was treated with thymidine and then exposed to TTFields. TTFields-exposed cells that were not treated with thymidine were used as control.

After thymidine treatment, the apoptosis of U373 cells exposed to TTFields was analyzed via annexin-V staining. The rate of apoptosis in U373 cell line untreated with thymidine was increased (approximately 10-fold) upon TTFields, but the apoptosis rate in thymidine-treated U373 cells was not significantly changed (Fig. 3a, b). These results are very consistent with the results of a previous study, which reported that TTFields induces cell death through anti-mitotic effects (Giladi et al. 2015).

Next, we immunostained thymidine-treated or -untreated U373 cells for γH2AX to assess for DNA damage. Without thymidine treatment, the number of γH2AX foci was significantly increased (Fig. 3d) compared with the number in the control, but the number of the foci in the thymidine-treated cells was not changed (Fig. 3c).

Taken together, these results showed that U373 cancer cells treated with thymidine are not influenced by TTFields, suggesting that TTFields therapy may not be effective by itself when the cell cycle is arrested, such as in cancer stem cells (CSCs), which are known to exist within tumors.

TTFields causes DNA damage and apoptosis in rapidly dividing cells

Next, to examine whether the effects of TTFields after cell-cycle arrest involve genes related to DNA damage or the cell cycle, such as BRCA1, PCNA, CDC25C and MAD2, we performed RT-qPCR analysis (Fig. 4).

After TTFields, the mRNA levels of all these genes strongly increased in U373 cells untreated with thymidine. TTFields had no significant effects in U373 cells treated with thymidine or in healthy HaCaT cells.

These results demonstrate that TTFields directly affects the expression patterns of genes related to DNA damage or the cell cycle. Therefore, identification of any factor (e.g., thymidine) that arrests the cell cycle may be very useful in protecting healthy cells against DNA damage and apoptosis when TTFields is used in clinical applications in the future.

Discussion

In this study, we demonstrated that TTFields inhibited the cell growth in our well-established TTFields system, indicating that this strategy targets the cell cycle. Additionally, we observed in cancer cell lines that thymidine treatment decreased the DNA damage and apoptosis caused by TTFields, and this observation was supported by the expression levels of genes related to cell cycle or DNA damage. These results suggest that the factors that prevent the DNA damage and apoptosis caused by TTFields should be identified for a safe cancer therapy in the future.

The adult healthy keratinocytes were not affected by TTFields with the adequate range of intensity (Fig. 1). However, the intensities of the TTFields on cancer cell lines decreased, as shown in our previous studies (Jeong et al. 2014), indicating that our TTFields system is well-established.

We showed that TTFields after thymidine treatment increased the colony formation abilities of cancer cell lines (Fig. 2). Thymidine-induced cell-cycle arrest had no effect on the apoptosis or DNA damage of the cancer cell lines (Fig. 3). These results suggest that TTFields targets a key factor in the cell cycle, and thymidine treatment protects

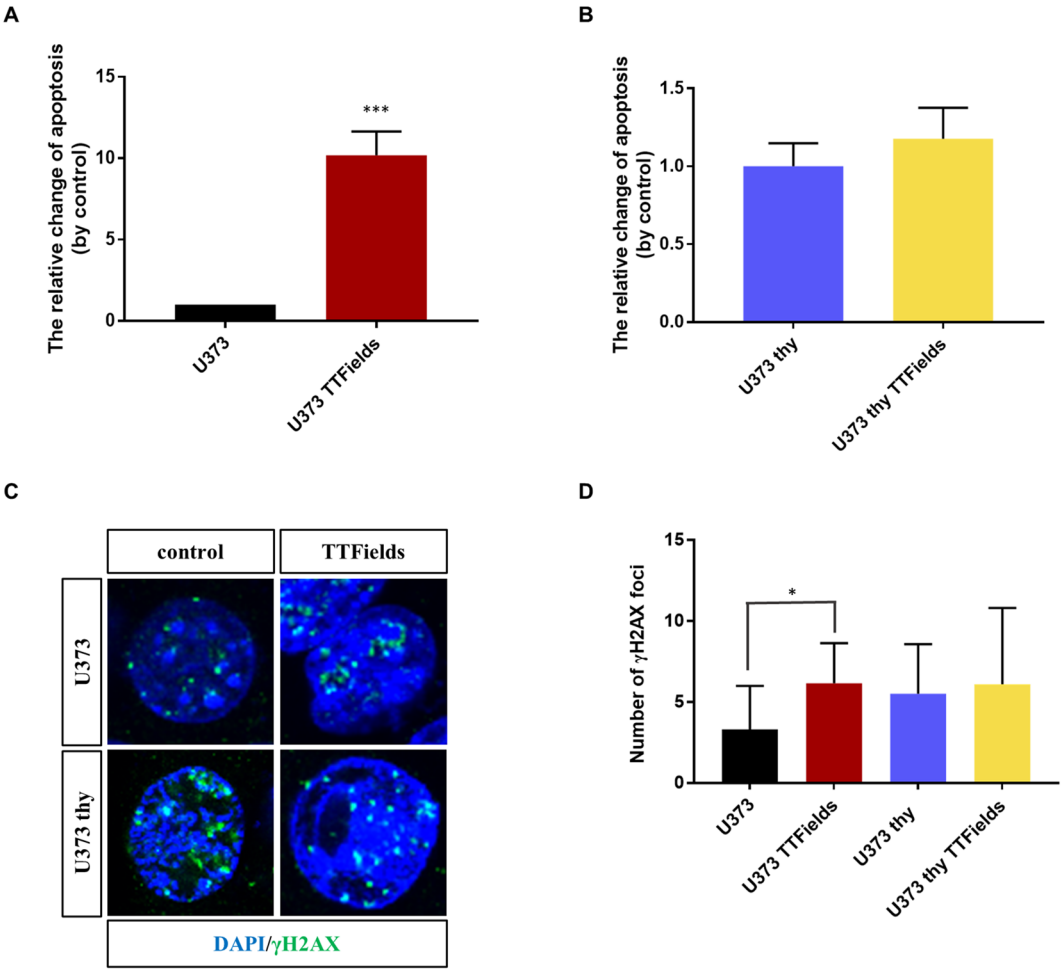

Fig. 3 Inhibition of the DNA damage and apoptosis in TTFields-treated cancer cells by thymidine. **a** The ratio of apoptosis in U373 cancer cell line untreated with thymidine. **b** The ratio of apoptosis in U373 cancer cell line treated with thymidine. **c** Representative γH2AX–immuno-staining images of the cancer cell lines treated with or without thymidine. **d** The number of γH2AX foci in the cancer cell lines treated with or without thymidine. *< 0.05, **< 0.005, ***< 0.0005

healthy cells from the DNA damage and apoptosis caused by TTFields.

Previous studies have reported that thymidine treatment modifies the phosphorylation on Ser139 of histone H2AX, but TTFields itself did not increase the DNA damage or apoptosis in the thymidine-arrested cells in our current study, demonstrating that TTFields has no influence on non-dividing cells.

The expression levels of specific genes related to the cell cycle or DNA damage, such as BRCA1, PCNA, CDC25C and MAD2, were not increased in the keratinocytes or the thymidine-treated cancer cell line, compared with the levels in the cancer cell line untreated with thymidine. These observations suggest that TTFields influences dividing cells (Fig. 4). Wang et al. have reported that BRCA1 and PCNA are involved in the recognition and repair of aberrant DNA structures (Wang et al. 2000). CDC25C participates in regulating G2/M progression and mediating DNA-damage repair as a cyclin of the specific phosphatase family that activates the cyclin B1/CDK1 complex in cells entering mitosis (Liu et al. 2020). In addition, MAD2 prolongs the DNA-damage checkpoint arrest caused by a double-stranded DNA break (Dotiwala et al. 2010). The upregulation of these genes suggests that the TTFields-caused DNA damage may delay G2/M phase (Johnson et al. 2016).

CSCs retaining a quiescent slow-cycling phenotype (Chen et al. 2016; Moore and Lyle 2011) within solid tumors might not be affected by TTFields, because the effect of TTFields is dependent on cell division. CSC is a cancer cell with stemness properties, tumorigenic potential, and chemoresistance, and thus anti-cancer therapy using TTFields might require co-treatment with other conventional therapies, such as chemotherapy.

In conclusion, pre-thymidine treatment dramatically decreases the DNA damage and apoptosis caused by TTFields. These results suggest that combining conventional

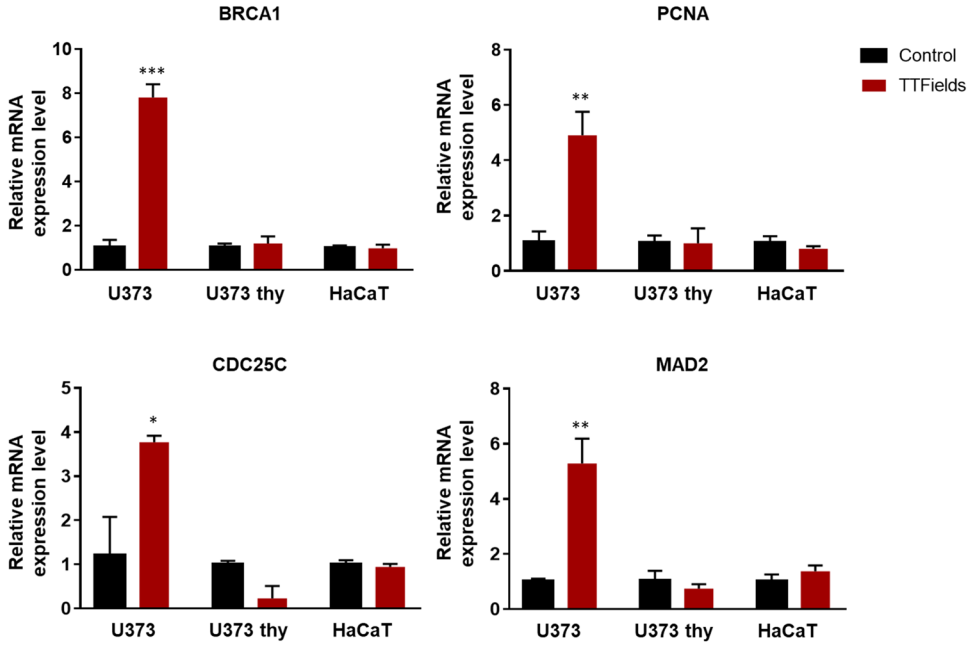

Fig. 4 The mRNA levels of genes related to DNA damage or the cell cycle. *< 0.05, **< 0.005, ***< 0.0005

chemotherapies with TTFields may greatly improve the prognosis in cancer while decreasing the treatment-associated morbidity.

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Declarations

Conflict of interest Hyesun Jeong, Yunhui Jo, Myonggeun Yoon, and Sunghoi Hong declare that they have no conflict of interest.

References

Chen W, Dong J, Haiech J, Kilhoffer MC, Zeniou M (2016) Cancer stem cell quiescence and plasticity as major challenges in cancer therapy. Stem Cells Int 2016

Dotiwala F, Harrison JC, Jain S, Sugawara N, Haber JE (2010) Mad2 prolongs DNA damage checkpoint arrest caused by a double-strand break via a centromere-dependent mechanism. Curr Biol 20:328–332

Giladi M, Schneiderman RS, Voloshin T, Porat Y, Munster M, Blat R, Sherbo S, Bomzon Z, Urman N, Itzhaki A et al (2015) Mitotic spindle disruption by alternating electric fields leads to improper chromosome segregation and mitotic catastrophe in cancer cells. Sci Rep 5:18046

Gonzalez CF, Remcho VT (2005) Harnessing dielectric forces for separations of cells, fine particles and macromolecules. J Chromatogr A 1079:59–68

Jeong H, Sung J, Oh SI, Jeong S, Koh EK, Hong S, Yoon M (2014) Inhibition of brain tumor cell proliferation by alternating electric fields. Appl Phys Lett 105:203703

Jo Y, Hwang SG, Jin YB, Sung J, Jeong YK, Baek JH, Cho JM, Kim EH, Yoon M (2018) Selective toxicity of tumor treating fields to melanoma: an in vitro and in vivo study. Cell Death Discov 4:46

Jo Y, Sung J, Jeong H, Hong S, Jeong YK, Kim EH, Yoon M (2019) Effectiveness of a fractionated therapy scheme in tumor treating fields therapy. Technol Cancer Res Treat 18:1533033819845508

Johnson C, Gali VK, Takahashi TS, Kubota T (2016) PCNA retention on DNA into G2/M phase causes genome instability in cells lacking Elg1. Cell Rep 16:684–695

Kim EH, Song HS, Yoo SH, Yoon M (2016) Tumor treating fields inhibit glioblastoma cell migration, invasion and angiogenesis. Oncotarget 7:65125–65136

Kim EH, Jo Y, Sai S, Park MJ, Kim JY, Kim JS, Lee YJ, Cho JM, Kwak SY, Baek JH et al (2019) Tumor-treating fields induce autophagy by blocking the Akt2/miR29b axis in glioblastoma cells. Oncogene 38:6630–6646

Kirson ED, Gurvich Z, Schneiderman R, Dekel E, Itzhaki A, Wasserman Y, Schatzberger R, Palti Y (2004) Disruption of cancer cell replication by alternating electric fields. Cancer Res 64:3288–3295

Kirson ED, Dbaly V, Tovarys F, Vymazal J, Soustiel JF, Itzhaki A, Mordechovich D, Steinberg-Shapira S, Gurvich Z, Schneiderman R et al (2007) Alternating electric fields arrest cell proliferation in animal tumor models and human brain tumors. Proc Natl Acad Sci USA 104:10152–10157

Kirson ED, Giladi M, Gurvich Z, Itzhaki A, Mordechovich D, Schneiderman RS, Wasserman Y, Ryffel B, Goldsher D, Palti Y (2009a) Alternating electric fields (TTFields) inhibit metastatic spread of solid tumors to the lungs. Clin Exp Metas 26:633–640

Kirson ED, Schneiderman RS, Dbaly V, Tovarys F, Vymazal J, Itzhaki A, Mordechovich D, Gurvich Z, Shmueli E, Goldsher D et al (2009b) Chemotherapeutic treatment efficacy and sensitivity are increased by adjuvant alternating electric fields (TTFields). BMC Med Phys 9:1

Liu K, Zheng MY, Lu R, Du JX, Zhao Q, Li ZG, Li YW, Zhang SW (2020) The role of CDC25C in cell cycle regulation and clinical cancer therapy: a systematic review. Cancer Cell Int 20:1–6

Moore N, Lyle S (2011) Quiescent, slow-cycling stem cell populations in cancer: a review of the evidence and discussion of significance. J Oncol 2011

Park JI, Song KH, Jung SY, Ahn J, Hwang SG, Kim J, Kim EH, Song JY (2019) Tumor-Treating Fields Induce RAW264.7 Macrophage Activation Via NK-kappaB/MAPK Signaling Pathways. Technol Cancer Res Treat 18:1533033819868225

Stupp R, Taillibert S, Kanner AA, Kesari S, Steinberg DM, Toms SA, Taylor LP, Lieberman F, Silvani A, Fink KL et al (2015) Maintenance therapy with tumor-treating fields plus temozolomide vs temozolomide alone for glioblastoma: a randomized clinical trial. JAMA 314:2535–2543

Stupp R, Taillibert S, Kanner A, Read W, Steinberg D, Lhermitte B, Toms S, Idbaih A, Ahluwalia MS, Fink K et al (2017) Effect of tumor-treating fields plus maintenance temozolomide vs maintenance temozolomide alone on survival in patients with glioblastoma: a randomized clinical trial. JAMA 318:2306–2316

Wang Y, Cortez D, Yazdi P, Neff N, Elledge SJ, Qin J (2000) BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. Genes Dev 14:927–939