RESEARCH ARTICLE

REVISED CCL2 and IL18 expressions may associate with the anti-proliferative effect of noncontact electro capacitive cancer therapy *in vivo* [version 2; peer review: 2 approved, 1 approved with reservations]

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Abstract

Background: Noncontact Electro Capacitive Cancer Therapy (ECCT) is a novel treatment modality in cancer. Chemokine (C-C motif) ligand 2 (CCL2) has a major role in the outgrowth of metastatic breast cancer. Interleukin 18 (IL18) plays a role in macrophage alteration, which leads to excessive angiogenesis. This study aims to elaborate on the association of CCL2, IL18, IL23α, and TNF-α (tumor necrosis factor-α) expression with the anti-proliferative effect of ECCT in rat breast tumor tissue.

Methods: Low intensity (18 Vpp) and intermediate frequency (150 kHz) alternating current-electric field (AC-EF) between two capacitive electrodes were exposed as external EF to a rat cage. Twenty-four rats were divided into four groups of six replicates. Breast tumor tissues were collected from 7, 12-dimethylbenz[a]anthracene (DMBA)-induced rats. Two groups were non DMBA-induced rats without ECCT exposure (NINT) and with (NIT). The other two groups were DMBA-induced rats without ECCT exposure (INT) and with (IT). Mammary glands and breast tumor tissues were collected from each group and preserved. Hematoxylin-eosin and immunohistochemistry staining were performed on paraffin sections of tissues using anti-PCNA, anti-ErbB2,

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anti-Caspase3, and anti-CD68. CCL2, IL18, IL23 α , and TNF- α mRNA relative expressions were analyzed using qRT-PCR.

Results: ECCT exposure may cause the reduction of PCNA protein expression as well as ErbB2 on breast tumor tissues, but it causes the increase of Caspase3 and macrophage CD68 protein. In rat breast tumor tissues of IT groups, the mRNA expression of CCL2 and IL18 are significantly down-regulated, in contrast with the up-regulated expression of these cytokines in tumor tissues of the INT group. IL23 α and TNF- α expression remained similar in both groups.

Conclusion: CCL2 and IL18 expressions have an association with the inhibition of breast tumor cell proliferation affected by ECCT exposure

Keywords

ECCT, rat breast tumor, anti-proliferative, IL18, CCL2

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REVISED Amendments from Version 1

The revised version includes edited introduction, methods, results and a figure. As suggested, the new citations from two references that related to the information have been added in the introduction. In the methods, some explanations have been edited especially for the use of sample replications and histological analysis. In the results, some explanations have been added and edited for clarity. Figure 4 has been edited to more clearly show the scale of relative expression.

Any further responses from the reviewers can be found at the end of the article

Introduction

Electro-Capacitive Cancer Therapy (ECCT) has been developed as a noncontact alternating current (AC) electric field (EF)-based cancer therapy method. A previous study¹ reported that ECCT low intensity (18 peak-to-peak voltage) intermediate frequency (100 kHz) treatment inhibits the growth of MCF-7 cells. Furthermore, this EF therapy could reduce the tumor size of C3H mice-breast tumor model, without abnormality in the dermal tissue and mammary glands of sham mice¹. Moreover, Mujib *et al.*² suggested that ECCT exposure (100–200 kHz) might induce p53 expression in cancer cells, such as oral squamous cell carcinoma, HeLa, and bone marrow mesenchymal cells. A previous study, using Tumor Treating Electric Field (TTFields) demonstrated that AC-EF with low intensity and intermediate frequency exposure to tumor cells caused the failure of tumor cell division toward the mitotic phase^{3,4}. A following study reported that the failure of tumor cell division was due to the disruption of spindle microtubule assembly, but not in normal cells⁵. From those studies, it was suggested that the failure of tumor cell division activates tumor cell apoptosis. However, the evidence that AC-EF exposure disturbs cancer cell proliferation and the molecular mechanism underlining this cell disturbance and elimination remains unknown.

Solid tumor growth and development are dependent on inflammatory cells in its microenvironment. Stromal components, such as endothelial cells, myeloid derivate suppressor cells, and macrophages, reside in the solid tumor microenvironment⁶. Macrophages act as inflammatory cells that can be affected by chemical signals, i.e. cytokines and chemokines^{7,8}, and electric fields⁹. A previous study reported that CCL2 is a chemo-attractant that binds to its receptor (CCR2) on monocytes, macrophages, and lymphocytes. In a previous *in vivo* study, CCL2-induced chemokine cascade in macrophage-associated metastasis (MAM) produced another ligand, CCL3, for metastatic seeding of breast cancer cells^{7,10}. In addition, IL18 plays a role in the migration of breast cancer cells via down-regulation of claudin12 and p38 MAPK (mitogen activation kinase) pathway¹¹. Hoare *et al.*⁹ demonstrated that electrical signals have been identified as major contributors to the coordination and regulation of macrophage functions. So far, electric fields-based therapies need to be described in order to understand the modulation of macrophage function, which underline the solid tumor microenvironment. The mechanism needs to be investigated.

Tumor necrosis factor-alpha (TNF)- α cytokine is one member of the tumor necrosis factor superfamily with a wide spectrum of biological activity. Menegatti *et al.*¹² reviewed TNF- α early on, and this inflammatory cytokine was suggested to be a potential antitumor agent and inducer of apoptosis *in vitro*. However, in the following years, recent studies reported that TNF- α significantly induces breast cancer metastasis via TNF α -activated mesenchymal stem cells (MSCs) in a lung metastasis model of murine breast cancer^{13–15}. However, under TNF- α stimulation at *in vitro* model, the MSCs can also release IL-10 as anti-inflammatory cytokine that may be a beneficial for cancer growth inhibition¹⁶. Although many studies focus on the function of TNF- α in the solid tumor microenvironment, the function remains as yet not fully clarified. So far, we understand that macrophages are multifunctional in the solid tumor microenvironment. Tumor-associated macrophages (TAMs) help tumor cell growth by releasing several pro-inflammatory cytokines, such as TNF α and IL23¹⁷. A previous study suggested that IL-23 is involved in inflammation and angiogenesis activities in the tumor microenvironment in spite of moderating CD8⁺ T-cell infiltration¹⁸. However, recent study suggested that TAM is an activated M2 macrophage¹⁹. Furthermore, the evaluation of IL-23 suggested that this cytokine has a function in promoting tumor metastasis and growth by upregulating matrix metalloproteinase (MMP)-9²⁰. On the other hand, Zimolag *et al.*²¹ reported that direct current (DC)-EF in the physiological condition might reposition MCSs into a wound site and allow macrophages to be at a short distance to the wound. Therefore, the expression of either TNF α or IL-23 cytokines-produced inflammatory cells in the microenvironment of solid breast tumor during the physiological DC-EF or AC-EF need to be further examined.

The anti-proliferative effect of AC-EF has been reported using several cell lines and in some tumor animal models. As reported by Ma *et al.*²², DMBA-induced breast cancer enhanced chromosomal instability and increased ErbB2-mediated mammary carcinogenesis. However, the association and mechanism of killing tumor cells and how the immune system clears death cells needs to be further clarified. A recent study demonstrated that TTFields therapy activates macrophage specific immune responses through the activation of several cytokines, such as TNF- α , and IL1- β *in vitro*¹⁰. In the present study, we elaborate on the relationship between the anti-proliferative effect of ECCT on DMBA induced-rat breast cancer cell growth and on the activity of inflammatory cells to express cytokines IL18, TNF- α , IL23 and chemokine CCL2, which play an essential role to the development of solid breast tumors. The use of this breast tumor animal model in this study is needed, because this model can represent the condition of breast cancer in patients. The main objective of this study is to examine the effectiveness of ECCT treatment on tumor growth inhibition through the molecular communication among stromal cells in the solid microenvironment tumor.

Methods

Animals

This study was carried out at the animal house of LPPT Research Center Universitas Gadjah Mada (UGM) which is

accredited by ISO/IEC 17025:2000 (a laboratory management standard). All requirements for animal welfare following the LPPT Ethics Committee Guidance have been fulfilled. This animal experiment has been legalized with an Ethical Clearance certificate number: 00029/04/LPPT/2018. The rat number for this experimental design was calculated for the minimal biological replication of rats ($n=6$), with four treatment groups according to the Federer Formula²³. 24 female rats (*Rattus norvegicus* Berkenhout, 1769) Sprague Dawley (SD) strain, five weeks old and weighing 50–80 grams were used in this study. The rats were obtained from LPPT Research Center.

Rats were fed with AIN-93M standard diet and standard water *ad libitum*. Rats were placed in a standard animal room (temperature and humidity were 20 to 25 °C and 40 to 60%, respectively). Rats were acclimatized to the laboratory condition and standard cage for 5 days.

This study focused on samples of rat breast tumor and healthy mammary gland. Therefore, we only observed the minimal number of tissue samples required for replication; three tissues samples ($n=3$) per treatment group (4 groups) was used for biological replication. Each sample was measured twice for qRT-PCR analysis and triple sections per tissue sample for histopathologic scoring requirements. Each rat was marked individually using picric acid (non-toxic) staining. Rats were observed for behavior, general physical conditions such as hairs, eyes, nose, ears, and feces every day. Rats were weighed every three days during the experiments using the balance scale for rodents. Rat welfare was maintained following the standard protocol from LPPT Research Center.

The experimental design of ECCT exposure treatments used four rat groups, six biological replicates, which consisted of:

- NINT group: non-DMBA-induced rats, non-EF therapy exposure
- NIT group: non-DMBA-induced rats, EF therapy exposure
- INT: DMBA-induced rats, non-EF therapy treatment
- IT: DMBA-induced rate, EF therapy exposure

Rats were induced ten times with DMBA^{24,25} doses of 20 mg/kg body weight within five weeks by oral administration. The DMBA (Sigma Aldrich; cat. no. D3254-1G) administrations were done around 04 p.m. in the animal room by the technician of LPPT Research Center following the Standard Operational Procedure (SOP) for DMBA treatment animal. After DMBA administration, all rats were palpated every two days using the standard procedure of palpation from LPPT Research Center. The tumor nodule was observed around 4 to 6 weeks after DMBA administration. Tumor nodule diameters were measured every 2 days using a digital caliper (Fisher Scientific) and all data measurements were tabulated.

Solid tumor (± 1 cm tumor size)-bearing rats were exposed to an AC-EF of 150 kHz and low intensity of 18 Vpp. EF therapy was performed for 21 days, with a total exposure of 10 hours per day with 2 hours rest after first 5 hours exposure. The

starting time of ECCT treatments were at 06 to 11 a.m., then at 01 to 06 p.m. During ECCT-exposing in the individual ECCT cage (designed by Ctech Labs Edwar Teknologi, IDN Patent REG. P00201200011), rats were fed with a standard diet and cucumber *ad libitum*, however during rest hours, rats were fed with a standard diet and water *ad libitum* in a communal cage with standard bedding and feeding for 5 rats. The ECCT treatment was finished after 21 days of treatment. Rats were sacrificed (euthanasia) by ketamine hydrochloride (KETALAR® Pfizer; cat. no. 629-24006) injection with a dosage of 150mg/kg body weight on the day after the last treatment. Rats were sacrificed starting at around 08 a.m. with the standard ethics procedure for rat euthanasia and surgery. After taking the samples, the remaining dead rat bodies were put in the freezer prior to eradication of the carcinogenic (DMBA) contaminated animals using the SOP of the LPPT animal house. Mammary glands and solid tumor tissues were sliced and fixed in 10% NBF (neutral buffer formalin, Bio-Optica; cat. no. 05-K01004) with ratio 5:1 for histological examination and in RNAlater® (Invitrogen; cat. no. AM7024) solution for total RNA extraction.

Histological examination

Mammary glands and solid tumor tissues were fixed in NBF and then processed using the paraffin method and stained with hematoxylin-eosin using the procedures provided by Bancroft and Cook²⁶. Summarily, the samples were periodically washed with 70% alcohol and subsequently dehydrated using a higher concentration of alcohol (80–100%). The dehydrated samples were then cleared with toluene (Merck; cat. no. 1083252500) overnight. The samples were infiltrated with paraffin (Merck; cat. no. 1073372500) in a 65°C oven and then embedded with freshly prepared paraffin. The sample paraffin blocks were sectioned with a microtome (Microm HM 315) providing a 4–6 μ m thick slice, which were then placed on a slide. Later on, the samples were then deparaffinized using xylene (Merck; cat. no. 1086612500), rehydrated using a downgraded concentration of alcohol (96–40%), and finally stained with Hematoxylin (made from Hematoxylin Krist C.I.75290, Merck; cat. no. 1159380025, using Erlich's formulation) and Eosin solution (made from Eosin Y, CI. 45380, Merck; cat. no. 1159350025). The stained samples were subsequently dehydrated using an upgraded level of alcohol, cleared in xylene, and lastly, mounted with Entellan (Merck; cat. no. 1079600500) and coverslip. The random 50 fields of view on IHC slides of each treatment were observed under Leica ICC50 E at 0.5 μ m/pixel resolution

Immunohistochemistry

The 4–6 μ m thick paraffin section of samples were placed on a Poly-L-lysine coated slide. The INT and IT tumor tissue samples were then processed using the Starr Trek Universal-HRP Detection Kit (Biocare Medical; cat.no BRR 700 AH, AL10) using the manufacturer's protocols. In brief, the samples were deparaffinized using xylene and then rehydrated with down-graded concentration of alcohol. The samples were heated in the microwave with sodium citrate buffer pH 6.0 for antigen retrieval for 15 minutes at 95 °C. The samples were soaked with 3% H₂O₂ (Sigma-Aldrich) in PBS for 5 min to block endogenous peroxidase and subsequently treated with

Background Sniper for 20 minutes for suppressing nonspecific binding. Afterwards, samples were separately incubated with anti-PCNA (ABCAM; cat.no. ab18197), anti-caspase-3 (ABCAM; cat.no. ab13847), anti-CD68 (ABCAM; cat.no. ab201340), and anti-ErbB 2 (ABCAM; cat.no. ab16901) antibodies overnight at 4 °C, followed by Trekkie Universal Link incubation for 60 minutes. Then, the samples were incubated with Trek-avidin HRP Label for staining development and then counterstained with hematoxylin. Lastly, the samples were dehydrated using an upgraded concentration of alcohol, cleared with xylene, and mounted with Entellan and coverslip. Immunohistochemistry slides were observed under Leica ICC50 E at 0.5 µm/pixel resolution with 50 fields of view.

Quantitative-RT-PCR

Quantitative-RT-PCR (qRT-PCR) was applied for measuring the transcriptomic expression (mRNA) of IL18, CCL2, TNF- α , and IL23 α (due to the use of sub unit p19 fragment for primer construction) genes. Isolation of RNA was performed using Total RNA Mini Kit (Blood/cultured cell; Geneaid; cat.no RB100), and cDNA synthesis with Superscript[®] III first-strand synthesis supermix (Invitrogen; cat.no 18080-400). Primers were as follows:

- IL23 α NM_130410.2 F: CAGGTTCCCATGGCTACAGT, R: TCT GGGGTTTGTGTGCTTTTC
- IL18²⁷ F: CAGACCACTTTGGCAGACTTCA, R: ACACAGCGGGTTTCTTTTGT
- TNF- α ^{27,25} F: AGCATGATCCGAGATGTGGAA, R: AATGAGAAGAGGCTGAGGCACA
- CCL2²⁸ F: 5' GTGCTGTCTCAGCCAGATGCAGTT 3', R: 5' AGTTCTCCAGCCGACTCATTGGG 3'.
- GADPH²⁹ F: 5' TGACAACTTTGGCATCGTGG 3', R: 5' GGGCCATCCACAGTCTTCTG 3'

RT-PCR analysis was performed using Universal SYBR[®] Green Supermix paint SsoAdvanced (BIO-RAD; kit. No. 172-5270). The thermal cycling conditions for amplification of IL18, TNF α , IL23 α , CCL2 and GADPH were the same for pre-denaturation at 95°C, 30"; and denaturation at 95°C, 10".

However, the annealing conditions were different: IL18 TNF α , and GADPH, 60°C for 10"; IL23, 60°C for 15"; and CCL2, 65°C for 10". All RT-PCR reactions were done using a BIO-RAD CFX96[™] Real-Time System, C1000 Touch[®] Thermal Cycler machine. qRT-PCR data was calculated using the Livak method³⁰.

Statistical analysis

Data analysis for IHC was scored automatically using color deconvolution and computerized pixel profiling by IHC Profiler plugin on ImageJ version 1.51 software³¹.

The independent-T test with IBM SPSS Statistics v22 was used for image scoring between groups and for qPCR data analysis. All graphs in this article were created by GraphPrism 7 software.

Results

Solid tumor growth

The rats prior and during ECCT treatment did not have pathogen infection, as observed during daily observation. During the experiments, the rats' body weight for all groups was not significantly different with the rat base line or untreated rats (secondary data not shown). Results of ECCT-exposed rat breast tumor (IT) show that the increase of the fluid bathing the tumor may affect the tumors size. This is in contrast with the results from the sham rat breast tumor group (INT), which exhibited denser tissues inside the solid tumor and slower growth (Figure 1).

Histopathological examination

Based on histological sections using hematoxylin-eosin staining (Figure 2), the observation of ECCT exposure effect on average rat mammary glands shows that there is no morphological tissue damage in both treatments, NINT nor NIT rat groups (Figure 2A and B). In solid tumor tissue sections, DMBA-induced tumor cells grew massively in both treatments (Figure 2C, D, E and F). Decreasing of fat and myoepithelial cells, and increasing of necrotic cells in mammary tissues due to the high tumor cell proliferation activity and minimal blood supply for healthy cells³². The lumens of mammary glands were filled with massive proliferative cells (Figure 2 C, D, E and F), in contrast with the healthy mammary gland which

Figure 1. Growth of solid breast cancer mass after ECCT exposure treatment. (A) Representation of solid tumor mass after surgery and (B) the solid breast tumor growth curve based on the diameter of tumor nodules. Tumor growth measurements were taken every two days for 21 days. INT= DMBA-induced rats without EF therapy, IT= DMBA-induced rats with EF therapy exposure.

Figure 2. Histological sections of the mammary gland and DMBA-induced rat breast adenocarcinoma after treatment with ECCT exposure. (A and B) There is no morphological change both on mammary gland tissue of none DMBA-induced rat non-EF therapy (NINT; A), and with EF therapy (NIT; B). Hematoxylin and eosin staining, magnification 100x. (C and D) In contrast, adenocarcinoma breast tissue of DMBA induced rat non-EF therapy (INT; C) shows more massive tumor cells and fewer lumens than the tumor section with EF therapy (IT; D). Hematoxylin and eosin staining, magnification 400x. (E and F) Mitotic figure (black arrow) and apoptotic figure (yellow arrow) on breast tumor tissues of INT and IT group, respectively. FC= Fat Cells, L=Lumens, EC=Epithelial Cells, M= Myoepithelial Cell, NA= Necrotic Area, BV= Blood Vessels.

contain a layer of epithelial cells (Figure 2 A and B). According to Denisov *et al.*³³, breast tumors develop morphological diversity related to the tumor progression. The morphological tumor cell growth pattern, such as solid tumor, reveals tens to hundreds of groups of shapeless tumor cells. This solid tumor cell pattern is related to tumor invasion or bad prognosis. The tubular structures or tube-shaped pattern of tumor growth is more similar with normal tubular mammary ducts³³. Figure 2 C and E (INT group) demonstrated a more solid tumor and less tubular tumor pattern. In contrast, the tubular tumor structure was observed more frequently in the IT group (Figure D and F) than in INT group. In general, tumor growth patterns of both INT and IT groups have solid tumor morphology. Therefore, those tumor can be identified as breast adenocarcinoma³³. The breast tumor cells grow invasively to other healthy tissues and caused necrosis area on both treatments, since the healthy myoepithelial and endothelial cells of blood vessels were

sited on the solid tumor sections. However, other tissues were removed from the observed-tumor tissue. On the sections of ECCT-treatment (IT), the invasive tumor cells showed a reduced number of mitotic cells and more apoptotic cells (Figure 2E and F). Moreover, it can be seen that lumen epithelial cells were more frequent for ECCT-exposure treatment than in tumor tissues without ECCT-exposure.

Immunohistochemistry quantification of protein expression on tumor tissues

Figure 3 shows the appearance of the protein of PCNA, ErbB2, Caspase 3, and macrophage CD68 on solid tumor tissues of ECCT treatment either with ECCT (IT) or without (INT). The percentage of tumor cells expressing PCNA protein in the IT group was significantly lower than in the INT group ($p < 0.01$). Consistent with PCNA expression, ERBB2 protein expression shows a significant decrease in tumor tissues of the IT group

Figure 3. Immunostaining of breast adenocarcinoma tissue sections after ECCT treatment. (A and B) Anti-PCNA, (C and D) anti-ErbB2, (E and F) anti-Caspase-3, (G and H) anti-CD68. Percentage of positive cells of (I) PCNA, (J) ErbB2, and (K) Caspase-3 in 50 fields of view. (L) Count of total macrophages in 50 fields of view. Observation of histological slide was performed using Leica CC50 E at 0.5 $\mu\text{m}/\text{pixel}$ resolution at 400x. Bar=100 μm . The mean, standard deviation of the data experiment show *, $p < 0.05$, **, $p < 0.01$. INT= DMBA-induced rats without EF therapy, IT= DMBA-induced rats with EF therapy exposure.

compared to the INT group ($p < 0.05$). In contrast, the appearance of Caspase 3 and CD68 proteins on tumor tissues of the IT group was significantly higher than the INT group ($p < 0.01$).

Relative mRNA expression of CCL2, IL18, TNF- α , and IL23 α genes

The relative mRNA expression of CCL2, IL18, TNF- α , and IL23 α genes can be seen in Figure 4. The appearance of

CCL2(15.29 fold change), was significantly lower ($p < 0.01$) on solid tumor tissues of the IT group than the INT group (97.72 fold change). This result was consistent with decreasing IL18 expression on tumor tissues of IT group (1.34 fold change) compared to the INT group (2.08 fold change). In contrast, the results of TNF- α and IL23 α expression in both groups was not significantly different. However, TNF- α gene expression was up-regulated relatively and IL23 α gene expression was

Figure 4. Relative mRNA expression of CCL2, IL-18, IL23α, and TNF-α in solid breast cancer after ECCT treatment. Amplification and melt peak chart of (A and B) CCL2, (C and D) IL18, (E and F) IL23α and (G and H) TNF-α. Relative expression of (I) CCL2, (J) IL-18, (K) IL23α and (L) TNF-α genes with NINT as a control group. The internal control of relative gene expression is GAPDH gene. (M) Relative expression of TNF-α, IL-23α, IL-18, and CCL2 INT vs. IT. Error bar shows a standard deviation of three replications; **p<0.01.

down-regulated relatively with the internal control of GAPDH gene expression. These results suggest that mRNA relative expression of CCL2 and IL18 were more down-regulated in tumor tissues exposed-ECCT (IT group) in comparison with the INT group. Whereas on the solid tumor tissues, the relative mRNA expression of both TNF- α and IL23 α genes were similar.

Discussion

DMBA-induced rat solid breast tumor has been studied in this study. Tumor interstitial fluid (TIF) formation could be affected by ECCT treatment, while other solid tumors not exposed to ECCT did not show a clearly visible effect. Although the substantial tumor size after ECCT exposure was not reduced, the solid tumor texture became soft and fluid (Figure 1A and B). According to Wagner and Wig³⁴, the TIF contains stromal and immune cells able to produce inflammatory substances into the solid tumor environment. TIF is an essential source of tumor-specific proteins that can be used to examine the effect of therapy on the mechanism of tumor-promotion or inhibition. Here we connected the TIF phenomenon and the growth of tumor cells after treating rats with AC-electrical fields exposed ECCT. A previous study⁵ using TTField (200 kHz) reported that this electric field treatment could influence not only tumor mitotic arrest and cell death, but also on cellular movement, infiltration and migration using *in vitro* and *in vivo* tests.

One of the critical modalities in cancer therapy is to minimize side effects resulting from the treatment. Our results (Figure 2A and B) demonstrated that ECCT exposure on non-DMBA induced rats showed healthy mammary gland tissue, similar to those not exposed to ECCT (NIT and NINT groups, respectively). Normal lobules consisted of an epithelial cell layer in both treatments and there was no tissue damage, such as an atrophic duct or other tissue injuries. In the rat solid tumor tissues we observed an abundance of proliferative cells that expanded to most areas of mammary glands. The changing of cell composition on tumor nodule is not clearly defined. However, the condition of solid tumors treated with ECCT was better with than without exposure (Figure 2C and D). Tumors treated with ECCT had a lower number (unseen data) of mitotic figures than the sham tumor treatment. Indeed, the apoptotic figures on tumor tissues with therapy were higher (unseen data) than non-therapy tissues (Figure 2E and F). Necrotic tissue areas were found in both treatments, but were more frequent in ECCT exposed rat breast tumor tissues than non-exposed. A previous study on tumor cells and its microenvironment interaction suggested that molecules and intermediate signaling substances play a role in controlling cell infiltration, survival, and apoptosis, which occur at the same time. So far, these molecules could be used as molecular targets for anti-cancer therapy modality³⁵.

Alamsyah *et al.*¹ reported a decrease in solid tumor size after exposure with 100 kHz AC-EF ECCT. But in this study, we obtain different results, where the tumor size did not decrease using 150 kHz with a similar treatment method. Indeed, tumor size was not significantly increased. ECCT and TTFields are noninvasive cancer treatment modalities based on AC-EF with an intermediate frequency (100–200 kHz) and low intensity, however, they differ in noncontact and direct contact on skin,

respectively, and wave type^{1,3}. A recent study¹⁰ reported that TTFields influence the activation of macrophage-specific immune responses through apoptotic bodies of dead tumor cells due to the agitation of cell mitosis by EF. These mechanisms should be further examined using molecular approaches, e.g. transcriptomics, to target proteins specifically affected by EF based cancer therapy.

Protein PCNA is frequently used as a cell proliferation marker. In this study, we evaluated the effect of ECCT treatment as an anti-proliferative agent using an intermediate frequency of 150 kHz. Previous studies examined the anti-proliferative effects using several types of cancer cell lines and measurement of tumor size *in vivo*^{1,2}. In the current study, anti-proliferative activity can be seen in Figure 3A, B and I, which revealed that ECCT exposure enables inhibition of breast tumor cell proliferation ($p < 0.001$). This result is consistent with previous findings^{1,2}. In addition, Giladi *et al.*⁵ exposed TTFields 150 kHz on non-small cell lung cancer cell lines and KLN205 squamous cell carcinoma in mice, which revealed inhibition of cancer cell viability and lung robust tumor growth due to the effect of AC-EF (TTFields) treatment. According to Ma *et al.*²², DMBA promotes ErbB2-mediated carcinogenesis via genomic instability. In the present study (Figure 3C, D and J), DMBA induced rats bearing solid breast tumors, which were exposed with ECCT treatment (IT), a significantly lower percentage of ErbB2 expressed cells were observed compared to the non-ECCT treatment (INT). Therefore, the present study supports previous results that suggested the anti-proliferative effect of AC-EF based tumor or cancer therapy (TTField and ECCT).

The most crucial evidence of cancer therapy is reducing tumor cell growth caused by cell death. Apoptosis is a necessary cell death process for inhibiting metastatic cancer³⁶. The present study shows that the percentage of breast tumor cells expressing caspase 3 (effector caspase) in the IT group is higher than in the INT group (Figure 3E, F and K). It can be suggested that ECCT exposure disturbs cell mitosis toward cell death through apoptosis, autophagy or necrosis. However, this result indicates that apoptosis via caspase 3 might be a predominant mechanism of breast tumor cell death caused by ECCT-treatment. This result is consistent with the effect of reducing cell proliferation (using PCNA cell proliferation marker) in the IT group. Mujib *et al.*² suggested that p53 might be involved in apoptosis induction in several cancer cell lines via a caspase-dependent apoptosis mechanism after exposure to ECCT. The results shown by the present study need to be confirmed with other protein markers involved in the tumor cell death mechanism.

The clearance of cell death debris is the final stage of apoptosis. In this process, it involves phagocytic cells, such as macrophages. The current study (Figure 3 G, H, and L) showed increasing expression of CD68 (a marker for macrophage and monocyte) on breast tumors exposed to ECCT (IT group), which was significantly higher than non-EF therapy (INT). Macrophages are multifunctional in solid tumor microenvironments, including macrophage polarization M1 and M2 which are involved in TAM and MAM functional directions^{7,37}. Ni *et al.*³⁸ suggested that the CD68 positive macrophage is one of the tumor-infiltrating

macrophages in non-metastatic breast cancer. Following the anti-proliferative effect of ECCT exposure, the up-regulation of classical CD68 macrophage marker may involve the expression of chemokine related macrophage polarization. This argument is supported by Park *et al.*¹⁰, who reported that TTFs-induced inflammatory action is via the p38 MAPK/NF- κ B signaling pathway and they believe that AC-EF based tumor therapy is a novel anticancer modality.

Solid tumor interstitial fluid is an excellent environment for communication of signaling substances among stromal, macrophages and tumor cells. In this study (Figure 4), we evaluated the expression of CCL2, IL18, TNF- α , and IL23 α , and whether they play a role in tumor progression or suppression. We demonstrated that the monocyte chemoattractant protein 1 (MCP-1) or CCL2 expression in solid breast tumor tissue with ECCT exposure (IT) is significantly lower (down-regulated) than without exposure (INT). There was no increase of relative mRNA CCL2 expression on ECCT exposure to the mammary gland of none DMBA induction rats (NIT) is shown in Figure 4I. This result supports a previous study that suggested that CCL2 has an essential role during breast cancer progression, through the induction of a systemic neutrophilic inflammatory cascade to facilitate metastasis³⁹. Moreover, Lavender *et al.*⁴⁰ reported that intra-tumoral CCL2 enables induction of breast tumor growth and/or metastases in breast cancer metastasis to the lung *in vivo*. Kirson *et al.*⁴¹ reported that TTFs (100 kHz and 1-2 Vpp) exposure has a potential action to inhibit the development of lung cancer metastasis from primary cancer cells. Additionally, IL18 and IL10 act synergistically on angiogenesis progression⁸. Following CCL2 expression, the present result demonstrated that IL18 expression is downregulated in breast tumor tissue exposed to ECCT treatment (Figure 4C, D, J, and M). The high expression of IL18 in breast cancer enables promotion of cell proliferation and migration, and could be a biomarker candidate for prognosis in breast cancer patients⁴². Indeed IL-18 expression leads to invasion and metastasis of breast cancer cells through PI3K-AKT/ ATF-2 signaling, and it might be regulated through NF- κ B/NF- κ B1 signaling in TAMs⁴³. Therefore, our result suggested that decreasing CCL2 and IL18 expression in the breast tumor microenvironment is most probably due to AC-EF treatment. We propose that there is a high correlation between the down-regulated expression of CCL2 and IL18 with the anti-proliferative effect of ECCT treatment. However, this argument should be further clarified by using other inflammatory markers of breast tumor progression.

In contrast with CCL2 and IL18 expression, in this study (Figure 4M) the appearance of IL23 α and TNF- α remains

unchanged with the relative expression of both cytokines in normal mammary glands (NINT and NIT groups) and both solid breast tumor (INT and IT groups). A previous review⁴⁴ reported that a higher TNF- α expression is related to breast tumor progression which is elevated in stage II, III and IV but not in stage I. IL23 is involved in inflammation and angiogenesis in response to cytotoxic T-cell infiltration in the tumor microenvironment. Therefore, in this study the relationship between IL23 α and TNF- α expression, and anti-proliferative effect of AC-EF based tumor therapy remain unclear.

Conclusions

In conclusion, we propose that noncontact ECCT treatment could have an anti-proliferative effect on solid breast tumor via the down-regulated expression of CCL2 and IL18. This preclinical study may become a basis of consideration for a clinical trial toward the implementation of ECCT as a novel cancer therapy modality.

Data availability

Underlying data

Open Science Framework: CCL2 and IL18 expressions may associate with the anti-proliferative effect of noncontact electro capacitive cancer therapy *in vivo*. <https://doi.org/10.17605/OSF.IO/EP7KT>⁴⁵.

This project contains the following underlying data:

- IHC data
- Nodul data
- Raw images for IHC and hematoxylin figures
- Rat body weights during treatment
- qPCR data

Data are available under the terms of the [Creative Commons Zero "No rights reserved" data waiver](#) (CC0 1.0 Public domain dedication).

Acknowledgments

We would like to thank M. Irfan, Finandya Fatihasari, Helmi Hana Prinanda, Ibnu Fajar, Miftah Jauhar, Puji Nurani, Windah Dwi Nuraini, Dalila Afinan, Ni Luh Kemmy Caesaria, Sevi Ratna Sari, Afif Yati and Pradana Putra Satya Negara for their kind helps during the works using animals, also many thanks to Mrs. Ardaning Nurliani for helping the antibodies procurement.

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Version 2

Reviewer Report 27 July 2020

<https://doi.org/10.5256/f1000research.28071.r67858>

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Agung Putra

Stem Cell And Cancer Research (SCCR), School of Medicine, Sultan Agung Islamic University (UNISSULA), Semarang, Indonesia

Regarding the revised article, entitled "CCL2 and IL18 expressions may associate with the anti-proliferative effect of noncontact electro capacitive cancer therapy in vivo", all of the corrections have revised completely. The revisions provide all aspects that need to be improved, according to reviewer expectation and meet the acceptable scientific standards. Therefore, reviewer has accepted this article for indexing.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Mesenchymal Stem Cell, Immunology, Cancer

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 09 July 2020

<https://doi.org/10.5256/f1000research.22795.r65163>

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¹ Stem Cell And Cancer Research (SCCR), School of Medicine, Sultan Agung Islamic University (UNISSULA), Semarang, Indonesia

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This manuscript addresses the effects of noncontact electro capacitive cancer therapy (ECCT) to down-regulate CCL2 and IL18 expressions in a breast cancer mouse model in which the end targets is inhibiting breast cancer cell proliferation. It is **an interesting topic** in efforts to eliminate cancer cells by elaboration between the anti-proliferative effect and activity of inflammatory cells following ECCT administration to breast cancer, due to the conventional therapy to reduce recurrence and breast cancer mortality **remains unclear**. On the other hand, the proinflammatory cells releasing proinflammatory cytokines such as IL18, TNF- α , IL23, and chemokine CCL2 also **play an essential role** in the development of breast tumors.

In this paper, there are a number of issues regarding the methods and analysis that need to be clarified and addressed.

Below are more specific comments by section:

Introduction:

1. Minor corrections:

add a punctuation mark "comma" in this sentence:

- Stromal components, such as endothelial cells, myeloid derivate suppressor cells, and macrophages"

add "the" in this sentences:

- Furthermore, the evaluation of IL-23 suggested that this cytokine has... "

Add "-" in this sentences:

- and growth by up-regulating matrix metalloproteinase...."

Add "a" in this sentences

- MCSs into a wound site and allow macrophages to be at a short distance to the wound..."

1. "Macrophages and tumor cells act as inflammatory cells that can be affected by chemical signals." Tumor cells is not inflammatory cells.

2. So far, electric fields-based therapies need to be described in order to understand the modulation. The mechanism need to be investigated.

3. "TNF- α significantly induces breast cancer metastasis via TNF α -activated mesenchyme stem cells (MSCs) in lung." However, under TNF- α stimulation at invitro model, the MSCs can also release IL-10 as anti-inflammatory cytokine that may be a beneficial for cancer growth inhibition.

Please read this article:

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Method:

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Add “this” in the sentence:

- The rat number for this experimental design was calculated for the minimal biological replication of rats.... “

Add “a” in the sentence:

- Tumor nodule diameters were measured every 2 days using a digital caliper”
- The sample paraffin blocks were sectioned with a microtome...”
- The stained samples were subsequently dehydrated using an upgraded level of...”

Add “-” in the sentence:

Solid tumor-bearing rats were exposed....

Add “comma” in the sentence:

- using an upgraded level of alcohol, cleared in xylene, and lastly”

Remove “s” in the sentence:

- The INT and IT tumor tissues samples were then processed...”

Add “a” and “-” in the sentence:

- In brief, the samples were deparaffinized using xylene and then rehydrated with a down-graded concentration of alcohol....”
- 1. Please clarify the experimental group. The experimental design of this study used four rat groups with six biological replicates (n=6), however, the number of tissue sample required for replication is three tissues samples (n=3)...”

Results

1. The authors reported that ECCT-exposed rat breast tumor (IT) can induce the fluid bathing in tumors that associated with tumors size, in which the author's hypothesis is to inhibit breast tumor cell proliferation (anti-proliferation). Although, the results from the sham rat breast tumor group (INT) exhibited denser tissues and slower growth, however, the tumor volume of IT (treatment group) still exhibited larger than the sham (INT group), suggesting the result is on the contrary to the anti-proliferation. (Figure 1). Please clarify.
2. The authors declared that the percentage of cells expressing CD68 protein in treatment groups (IT) were significantly higher than in the non-treatment groups (INT group) indicated that the macrophage polarized into type-2, known as Tumor associated macrophage (TAM) that associated with the proliferation of cancer cell. Please clarify.
3. On the other hand, in this study there was a decrease of CCL2 as the main chemokine of

monocyte and macrophages, however the macrophage type-2 (CD 68 expression) was increased, Please clarify.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Mesenchymal Stem Cell, Immunology, Cancer

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 14 Jul 2020

Rarastoeti Pratiwi

External Reviewer (Dr. A. Putra):

APPROVED WITH RESERVATIONS

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- and growth by up-regulating matrix metalloproteinase...."
- and growth by up regulating

Add "a" in this sentences

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User comment:

All typo corrections have been done.

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User comment:

Revision has been done. In the new version article has been added "The mechanism needs to be investigated".

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User comment:

In the new version, a revision has been done by added a new reference: reference no 16.

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User comment:

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Revision has been done by deleting: "NF-kB (nuclear factor-kB),"

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Add "a" in the sentence:

- Tumor nodule diameters were measured every 2 days using a digital caliper"
- The sample paraffin blocks were sectioned with a microtome..."
- The stained samples were subsequently dehydrated using an upgraded level of..."

The stained samples were subsequently dehydrated using upgraded level

Add "-" in the sentence:

Solid tumor-bearing rats were exposed....

Add "comma" in the sentence:

- using an upgraded level of alcohol, cleared in xylene, and lastly"

using an upgraded level of alcohol, cleared in xylene and, lastly,

Remove "s" in the sentence:

- The INT and IT tumor tissues samples were then processed..."

Add "a" and "-" in the sentence:

- In brief, the samples were deparaffinized using xylene and then rehydrated with a down-graded concentration of alcohol...."

User comment:

In the new version, all these typo corrections have been done.

1. Please clarify the experimental group. The experimental design of this study used four rat groups with six biological replicates (n=6), however, the number of tissue sample required for replication is three tissues samples (n=3)..."

User comment:

Revision:

In the new version, the (n=3) have been deleted in the explanations of:"

- - NINT group: non-DMBA-induced rats, non-therapy exposure (n=3)
- - NIT group: non-DMBA-induced rats, therapy exposure (n=3)
- - INT: DMBA-induced rats, non-therapy treatment (n=3)
- - IT: DMBA-induced rate, therapy exposure (n=3)", because it could confuse.

In the new version, the replication of rat (n=6) and three tissues sample per treatment group (n=3) have been added in the text. So, the NINT, NIT, INT and IT just to explain the treatment for each group (without (n=3)).

Clarification:

As I described in the manuscript: "The rat number for this experimental design was calculated for the minimal biological replication of rats, with four treatment groups according to the Federer Formula ²³ " and this number also follow the ethical procedure for using the minimal animals. However, we only using a minimal number of tissue samples replication requirement for statistical analysis in random sampling method. Three tissues samples per treatment group (4 groups) was used for a biological replication requirement. Each sample was triple sections per tissue sample for histopathologic scoring requirements (to gain 50 fields of view) and twice for qRT-PCR analysis. However, we used six rats (n=6) per group to observe rat body weights (secondary data)

Results

1. The authors reported that ECCT-exposed rat breast tumor (IT) can induce the fluid bathing in tumors that associated with tumors size, in which the author's hypothesis is to inhibit breast tumor cell proliferation (anti-proliferation). Although, the results from the sham rat breast tumor group (INT) exhibited denser tissues and slower growth, however, the tumor volume of IT (treatment group) still exhibited larger than the sham (INT group), suggesting the result is on the contrary to the anti-proliferation. (Figure 1). Please clarify.

User comment:

Clarification:

The larger size of tumor nodules in IT groups due to the measurement using a digital caliper. In that case, we measured the nodule volume included the inflammation fluids, instead of tumor tissues. However, from the histological observation, the results showed that the number of proliferation cells in IT groups significantly lower than in INT group. So, from this result we suggest that the tumor interstitial fluid contains stromal and immune cells could increase the tumor nodule volume. Further, we observed the proliferation of the tumor cells (based on PCNA marker of cells), and the role of macrophage (based on CD68 marker of monocyte and macrophage) to eliminate the tumor cells which are failure to divided.

2. The authors declared that the percentage of cells expressing CD68 protein in treatment groups (IT) were significantly higher than in the non-treatment groups (INT group) indicated that the macrophage polarized into type-2, known as Tumor associated macrophage (TAM) that associated with the proliferation of cancer cell. Please clarify.

User comment:

Revision:

In the new revision, the words "a classical", in the sentence: "... expression of CD68 (a classical macrophage marker).." has been deleted and edited: "...expression of CD68 (a marker for macrophage and monocyte)

Clarification:

CD68 is the marker of macrophage and monocyte (see the revision above), so these macrophages could be M1 and M2 (TAM).

3. On the other hand, in this study there was a decrease of CCL2 as the main chemokine of monocyte and macrophages, however the macrophage type-2 (CD 68 expression) was increased, Please clarify.

User comment:

Clarification:

CD68 can be expressed either in monocyte and macrophage (M1 and M2). However, CCL2 level correlated with TAM abundance and TAM is M2 Macrophage. The results show that the increase of macrophage CD68 (could be M1 and M2 macrophages) in IT group, but the expression of CCL2 in this group is decrease.

- **Is the work clearly and accurately presented and does it cite the current literature?**

Yes

- **Is the study design appropriate and is the work technically sound?**

Yes

- **Are sufficient details of methods and analysis provided to allow replication by others?**

Partly

- **If applicable, is the statistical analysis and its interpretation appropriate?**

Partly

- **Are all the source data underlying the results available to ensure full reproducibility?**

Yes

- **Are the conclusions drawn adequately supported by the results?**

Yes

Competing Interests

No competing interests were disclosed.

Reviewer Expertise

Mesenchymal Stem Cell, Immunology, Cancer

I confirm that I have read this submission and believe that I have an appropriate level

of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Reviewer Report 05 March 2020

<https://doi.org/10.5256/f1000research.22795.r59978>

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Yoram Palti

¹ Novocure, Haifa, Israel

² Novocure, Haifa, Israel

The manuscript by Antara and colleagues reports on the use of noncontact ECCT treatment to down-regulated expression of CCL2 and IL18 and therefore inhibit breast cancer cell proliferation.

Comments:

1. The authors claim that the fluid bathing in breast tumors from ECCT-exposed IT treated rats may affect the tumors size, however, no specific analysis was done to support such claim. For instance, a single cell suspension of whole tumor followed by cell count could suffice. This data is crucial In order to support the claim that ECCT treatment is anti-proliferative, as tumor volume measurements show otherwise.
2. The authors should also elaborate on:
 - The impact of reduction in CCL2 and phenotype skewing in tumor macrophages.
 - The discrepancy between CCL2 levels and CD68 infiltration.
 - The discrepancy between past publications showing activation of the MAPK/NF-kB signaling pathway following AC-EF based tumor therapy (suggested as an example that has been used by the authors to support some of the conclusions in the current study) and the downregulation of IL18 in the current study.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: I am CTO of NovoCure

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 23 October 2019

<https://doi.org/10.5256/f1000research.22795.r55336>

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Richard Luke Daniels

¹ Department of Biology, The College of Idaho, Caldwell, ID, USA

² Department of Biology, The College of Idaho, Caldwell, ID, USA

This paper describes the effects of intermediate frequency (100-300 kHz) alternative electric fields in a mouse model of breast cancer. Specifically, the authors examined expression (by immunohistochemistry and/or qPCR) of several genes. These were immune factors known to be associated with tumor cell proliferation and angiogenesis (CCL2 and IL18), a marker of cell proliferation (PCNA), a growth factor receptor (ErbB2), a marker of leukocyte differentiation (CD68) several other immune factors (IL18, IL23-alpha, TNF-alpha), and a marker of apoptosis (caspase-3). Changes were seen in gene expression consistent with decreased cell proliferation and increased cell death, though pro-inflammatory changes were not seen. These results provide evidence that supports a biological effect of alternating electric fields on cell proliferation, and it is this reviewer's opinion that publication is warranted. There are a few minor modifications that I would suggest making before indexing this manuscript, which center around experiments described in figures 2-4.

Minor English language corrections:

In some instances, "none" should be changed to "non" throughout the document, as in "non DMBA-induced" (this is sometimes correctly stated, for example the animal methods section is correct).

In the conclusion, change “purpose” to “propose”

Methods:

It would be beneficial to give more specifics about the protein quantification done using ImageJ. Was the scoring automated in anyway? What was the process or procedure used for determining whether a cell was positive for a given marker?

Figure 2:

The figure supports what is described in the text; however no quantification is given regarding the stated observations regarding the cell composition of the tumor and the structural changes with and without treatment. I do not consider this to be essential for indexing, however it would strengthen the conclusions drawn from this experiment if these results could be quantified.

Figure 3:

The figure supports what is described in the text. See comments related to the scoring method above. In the statistics, it is a bit unclear as to what was counted as an individual n. Was each slide counted as a single n? Was each image (50 images / slide)? It would be helpful for context to describe in a bit more detail either in these results or the methods section what was counted as an individual experiment for interpretation of the statistical comparisons.

Figure 4:

This figure supports what is described in the text. The graphs would benefit from changing the Y-axes to be consistently labeled with the same amount of significant figures. Also – if the CCL2 expression is 100-fold different than NIT, etc., it might be beneficial to point this out in the discussion, as this increase is substantially more than other differences seen that are 2-fold different, for example.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

No source data required

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Cell physiology, Neuroscience, Cell signaling

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 14 Jul 2020

Rarastoeti Pratiwi

External Reviewer (Dr. R. L. Daniels):

This paper describes the effects of intermediate frequency (100-300 kHz) alternative electric fields in a mouse model of breast cancer. Specifically, the authors examined expression (by immunohistochemistry and/or qPCR) of several genes. These were immune factors known to be associated with tumor cell proliferation and angiogenesis (CCL2 and IL18), a marker of cell proliferation (PCNA), a growth factor receptor (ErbB2), a marker of leukocyte differentiation (CD68) several other immune factors (IL18, IL23-alpha, TNF-alpha), and a marker of apoptosis (caspase-3). Changes were seen in gene expression consistent with decreased cell proliferation and increased cell death, though pro-inflammatory changes were not seen. These results provide evidence that supports a biological effect of alternating electric fields on cell proliferation, and it is this reviewer's opinion that publication is warranted. There are a few minor modifications that I would suggest making before indexing this manuscript, which center around experiments described in figures 2-4.

Minor English language corrections:

In some instances, "none" should be changed to "non" throughout the document, as in "non DMBA-induced" (this is sometimes correctly stated, for example the animal methods section is correct).

In the conclusion, change "purpose" to "propose"

User comment:

All typo corrections have been done

Methods:

It would be beneficial to give more specifics about the protein quantification done using ImageJ. Was the scoring automated in anyway? What was the process or procedure used for determining whether a cell was positive for a given marker?

User comment

In the new version article has been revised: "Data analysis for IHC was scored automatically using color deconvolution and computerized pixel profiling by IHC Profiler plugin on ImageJ version 1.51 software ²⁹"

Figure 2:

The figure supports what is described in the text; however no quantification is given regarding the stated observations regarding the cell composition of the tumor and the structural changes with and without treatment. I do not consider this to be essential for indexing, however it would strengthen the conclusions drawn from this experiment if these results could be quantified".

User comment:

In the new version article has been revised:"The tumor nodule is not a complex tissue, usually consist of connective and alveolar tissues, therefore the changing of cell composition is not clearly defined".

Figure 3:

The figure supports what is described in the text. See comments related to the scoring method above. In the statistics, it is a bit unclear as to what was counted as an individual n. Was each slide counted as a single n? Was each image (50 images / slide)? It would be helpful for context to describe in a bit more detail either in these results or the methods section what was counted as an individual experiment for interpretation of the statistical comparisons.

User comment

In the new version article has been revised:"The random 50 fields of view on IHC slides of each treatment were observed under Leica ICC50 E at 0.5 μm /pixel resolution".

Figure 4:

This figure supports what is described in the text. The graphs would benefit from changing the Y-axes to be consistently labeled with the same amount of significant figures. Also – if the CCL2 expression is 100-fold different than NIT, etc., it might be beneficial to point this out in the discussion, as this increase is substantially more than other differences seen that are 2-fold different, for example.

User Comment

A revision has been done in the new version article:"The appearance of CCL2(15.29 fold change), was significantly lower ($p < 0.01$) on solid tumor tissues of the IT group than the INT group (97.72 fold change). This result was consistent with decreasing IL18 expression on tumor tissues of IT group (1.34 fold change) compared to the INT group (2.08 fold change)". Figure 4.I. has been edited for the scale of expression relative (fold change).

- **Is the work clearly and accurately presented and does it cite the current literature?**

Yes

- **Is the study design appropriate and is the work technically sound?**

Yes

- **Are sufficient details of methods and analysis provided to allow replication by others?**

Partly

- **If applicable, is the statistical analysis and its interpretation appropriate?**

Partly

- **Are all the source data underlying the results available to ensure full reproducibility?**

No source data required

- **Are the conclusions drawn adequately supported by the results?**

Partly

Competing Interests

No competing interests were disclosed.

Reviewer Expertise

Cell physiology, Neuroscience, Cell signaling

Competing Interests: No competing interests were disclosed.

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