Alternating Electric Fields (Tumor-Treating Fields Therapy) Can Improve Chemotherapy Treatment Efficacy in Non-Small Cell Lung Cancer Both In Vitro and In Vivo

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Non-small cell lung cancer (NSCLC) is one of the leading causes of cancer-related deaths worldwide. Common treatment modalities for NSCLC include surgery, radiotherapy, chemotherapy, and, in recent years, the clinical management paradigm has evolved with the advent of targeted therapies. Despite such advances, the impact of systemic therapies for advanced disease remains modest, and as such, the prognosis for patients with NSCLC remains poor. Standard modalities are not without their respective toxicities and there is a clear need to improve both efficacy and safety for current management approaches. Tumor-treating fields (TTFields) are low-intensity, intermediate-frequency alternating electric fields that disrupt proper spindle microtubule arrangement, thereby leading to mitotic arrest and ultimately to cell death. We evaluated the effects of combining TTFields with standard chemotherapeutic agents on several NSCLC cell lines, both in vitro and in vivo. Frequency titration curves demonstrated that the inhibitory effects of TTFields were maximal at 150 kHz for all NSCLC cell lines tested, and that the addition of TTFields to chemotherapy resulted in enhanced treatment efficacy across all cell lines. We investigated the response of Lewis lung carcinoma and KLN205 squamous cell carcinoma in mice treated with TTFields in combination with pemetrexed, cisplatin, or paclitaxel and compared these to the efficacy observed in mice exposed only to the single agents. Combining TTFields with these therapeutic agents enhanced treatment efficacy in comparison with the respective single agents and control groups in all animal models. Together, these findings suggest that combining TTFields therapy with chemotherapy may provide an additive efficacy benefit in the management of NSCLC.
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Lung cancer is the second most common cancer and the leading cause of cancer-related mortality in the United States.[1] It accounts for about 14% of all new cancers; more than 224,210 new cases are expected to be diagnosed in the United States in 2014.[1] Non-small cell lung cancer (NSCLC) accounts for 80% to 85% of all lung cancer cases. Most NSCLC patients present with advanced stage III or IV disease.[2] A common treatment modality for advanced NSCLC is cytotoxic chemotherapy, which is typically associated with a 20% to 35% response rate and a median survival of approximately 10 to 12 months.[3,4] For patients with advanced NSCLC who progress after receiving a platinum-based chemotherapy regimen, second-line treatments offer only modest survival benefit, often with significant toxicity.[5] Furthermore, patients with squamous histology (which accounts for ~25%–30% of all NSCLC cases) do not typically respond to newer targeted therapies,

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and managing these patients effectively remains an area of high unmet need.

Tumor-treating fields (TTFields) are a novel treatment modality delivered via continuous noninvasive application of low-intensity, intermediate-frequency alternating electric fields to the region of the tumor.[6] TTFields have demonstrated effectiveness in the treatment of solid tumors in vitro and in vivo.[6–10] Several pilot clinical trials and larger randomized studies in patients with solid tumors including glioblastoma have demonstrated the feasibility, safety, and effectiveness of continuous TTFields application in patients.[7] Of note, recent phase I/II trial showed, for the first time, preliminary efficacy and safety of combining TTFields therapy with pemetrexed in patients with stage IIIB/IV NSCLC following failure of standard first-line therapy.[11] This study demonstrated a median progression-free survival (PFS) of 22 weeks and an encouraging in-field PFS (measured at the site where TTFields were delivered to the lungs and liver) of 28 weeks. Median overall survival was 13.8 months and 1-year survival was 57%, both of which compared well to matched historical controls. These results support the clinical activity of TTFields therapy when added to chemotherapy in advanced NSCLC, without incremental toxicity.

The present study attempts to further elucidate the effects of TTFields in combination with different chemotherapeutic agents on several NSCLC cell lines, both in vitro and in vivo.

MATERIALS AND METHODS

Tumor Cell Lines

We obtained four human NSCLC cell lines and two murine cell lines from ATCC (American Type Culture Collection, Rockville, MD). The NSCLC cell lines were H1299 (adenocarcinoma), A549 (adenocarcinoma): HTB-182 (squamous cell carcinoma [SCC]), and HCC827 (adenocarcinoma, mutated in the epidermal growth factor receptor [EGFR] tyrosine kinase domain). The murine cell lines were LLC1 (Lewis lung carcinoma 1) and KLN205 (SCC). All human cell line cultures were maintained in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal calf serum (FCS). The LLC1 cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and 5% glutamine. The KLN205 cell line was maintained in MEM supplemented with 10% FCS. All cell lines were incubated in a humidified incubator supplied with 5% CO_2.

TTFields Application in Vitro

Methods for applying TTFields during the conduct of in vitro studies have been described previously.[7,9] In brief, two pairs of transducer arrays, insulated with a high dielectric constant ceramic (lead magnesium niobate–lead titanate), were positioned perpendicularly in a Petri dish. The transducer arrays were connected to a sinusoidal waveform generator at the desired frequencies in the medium. The TTFields changed direction by 90° every 250 ms. Temperature was measured by a thermocouple (Omega Engineering, Stamford, CT) placed in the center of the dish. TTFields were applied for 72 hours with or without the chemotherapeutic agents paclitaxel (Sigma-Aldrich, Rehovot, Israel), pemetrexed (Eli Lilly, Indianapolis, IN), cisplatin (Sigma-Aldrich), and erlotinib (LC Laboratories, Woburn, MA).

Cell Viability and XTT Assay

Inhibition of tumor cell growth was analyzed by quantitative determination of cell viability via standard XTT methods (Cell Proliferation Kit; Biological Industries, Beit Haemek, Israel). The cell number was determined and the relative number of viable cells at the end of 72 hours of treatment was normalized to the number of viable cells in untreated control.

Clonogenic Survival Assay

Cells treated for 72 hours with TTFields at 150 kHz frequency were subsequently harvested and replated into 6-well tissue culture plates (300 cells/well). Five to 10 days after seeding, colonies were stained with 0.5% crystal violet, and the number of colonies containing at least 50 cells were counted. Survival fractions were calculated relative to control.

Efficacy of Combination Treatments in Animal Models

All animal studies were approved by the Novocure Internal Animal Care committee in accordance with the Technion-Israel Institute of Technology guidelines for the care of laboratory animals. LLC1 cells (6×10^3) were mixed with 50% high-concentration growth-factor–reduced Matrigel (BD Biosciences, Bedford, MA) and were implanted in the left lung of 10-week-old C57Bl/6 mice (Harlan Laboratories, Jerusalem, Israel) 11 days before treatment initiation using the same method described by Onn et al.[12] Mice were randomly grouped (>25 per group) before treatment with TTFields, pemetrexed, cisplatin, paclitaxel, or TTFields combined with these agents. Mice were treated for 6 days using two-directional, 150-kHz TTFields at a field intensity of 1.86 ± 0.67 V/cm RMS. Two pairs of insulated transducer arrays were placed on the depilated chest skin overlying the lung area so that two perpendicular field directions were delivered sequentially (1 second in each direction) to the lungs. Treatment was applied for at least 90% of the duration of the experiment. TTFields parameters were continuously measured. The control

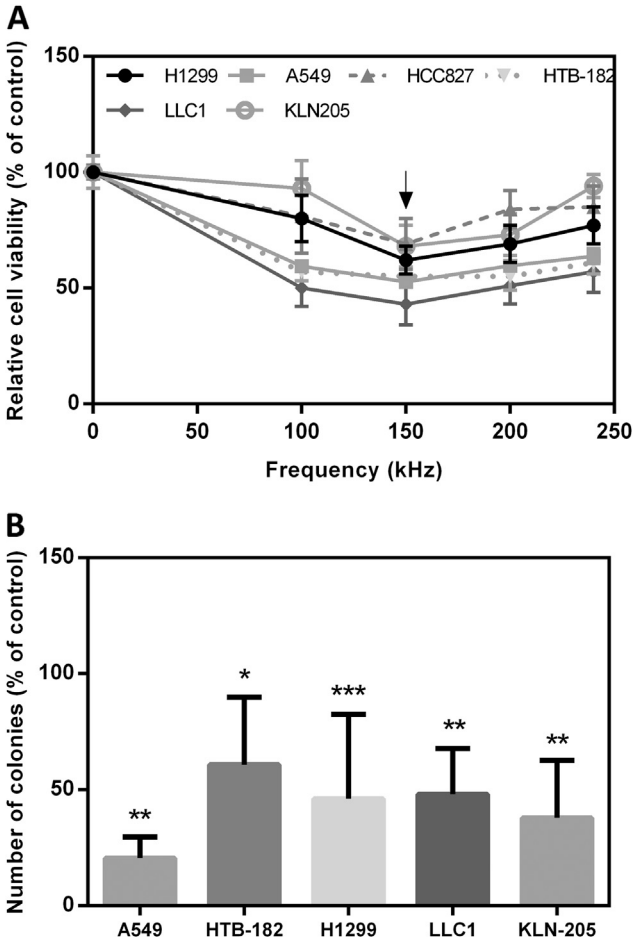

Figure 1. (A) Frequency-dependent effect of TTFields on viability of lung cancer cells and influence of optimal frequency on clonogenic potential. Effect of TTFields treatment, administered in different frequencies, on cell viability of various lung cancer cell lines (arrow indicates optimal frequency). (B) Clonogenic potential of various lung cancer cell lines after TTFields treatment at optimal frequency (150 kHz). $^{*}P<.05$,$^{**}P<.01$, and $^{***}P<.001$ versus control group.

and chemotherapy-treated mice had sham transducer arrays placed on their torsos; these arrays were temperature-matched to the TTFields transducer arrays.

Pemetrexed (1 mg/kg) and cisplatin (5 mg/kg) both (as single bolus injection), and paclitaxel (20 mg/kg twice a day every 3 days) were administered intraperitoneally on day 11 Eleven after tumor implantation, and immediately before TTFields application. Control mice received the relevant vehicles. Initial tumor volume was evaluated from lung samples from mice euthanized on day 1 of treatment initiation. Final tumor volume was assessed at necropsy (day 17 after tumor implantation) with Vernier calipers using the formula width^2 × length × 0.5.

The effects of TTFields on a rapidly growing, syngeneic orthotopic tumor model of DBA/2 mice injected with SCC were also examined. In brief, 2×10^4 murine SCC (KLN205) cells mixed with Matrigel were injected directly into the lungs of DBA/2 (Harlan) mice using the same method described for the induction of LLC1 orthotopic tumors. Tumor uptake was observed in 85%±15% of the mice 3 weeks after inoculation, but tumor progression was highly variable (average volume, 24 mm^3). Cells derived from DBA/2 lung tumors (KLN205-T1) were sub-cultured for more than 10 generations. KLN205-T1 cells were then thawed and cultured for 1 week before being used for inoculation. Mouse left lung lobes injected with 5×10^4 KLN205-T1 cells in 50% Matrigel demonstrated rapid tumor growth, with a mean volume of 51±13 mm^3 13 days after injection. During the same period, left lung lobe weight increased from 67±5 mg to 123±19 mg. The observed tumor uptake was 80%± 11%. Eleven days after tumor inoculation, the mice were randomly grouped (>12 per group) and treated with TTFields, cisplatin, or their combination, as described earlier.

Statistical Analysis

The statistical significance of differences were assessed with one-way analysis of variance followed by the Tukey range statistical test using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). Between-groups differences were considered significant at $^{*}P<.05$, $^{**}P<.01$, and $^{***}P<.001$. Data are expressed as means and standard deviations of the mean.

RESULTS AND DISCUSSION

TTFields Induce Frequency-Dependent Reduction in Viability and Clonogenic Capacity of NSCLC Cells In Vitro

The inhibitory effect of TTFields on tumor cell growth is well established in various tumor cell lines of different origins, but its specific impact on NSCLC cells remains unclear. We investigated the effect of TTFields in vitro, using four human NSCLC cell lines (H1299, A549, HCC827, HTB-182) and two murine cell lines (LLC1, KLN205). As the optimal TTFields frequency required to disrupt cell culture viability has previously been shown to be cell type–specific, the cells were exposed to various frequencies between 100 kHz and 240 kHz. TTFields therapy was found to induce a frequency-dependent reduction in viability in all six cell lines, with a common peak effective frequency at 150 kHz (Figure 1A). Interestingly, similar efficacy was observed for cultures of both adenocarcinoma and SCC histologies. In subsequent experiments, the clonogenic potential of single cells after 150-kHz TTFields treatment was compared with that of untreated cells. TTFields treatment was found to significantly impair the colony-forming ability of all tested cell lines in

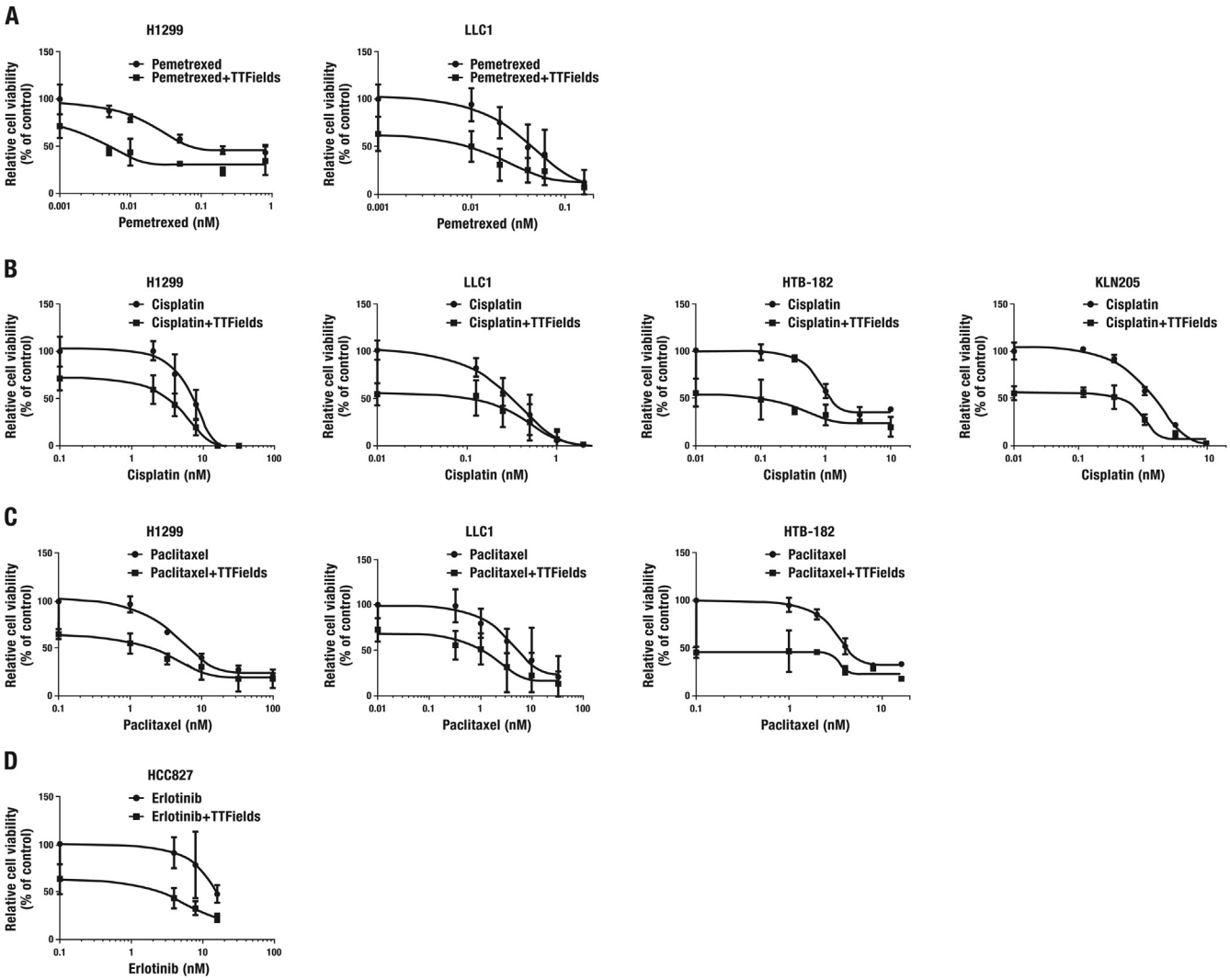

Figure 2. In vitro efficacy of chemotherapy and TTFields. Dose–response plots of (A) pemetrexed alone and in combination with TTFields on H1299 (adenocarcinoma) and LLC1 (Lewis lung carcinoma 1) cells; (B) cisplatin alone and in combination with TTFields on H1299, LLC1, KLN205 (squamous cell carcinoma), and HTB-182 (squamous cell carcinoma) cells; (C) paclitaxel alone and in combination with TTFields on H1299, LLC1, and HTB-182 cells; and (D) erlotinib alone and in combination with TTFields on HCC827cells (adenocarcinoma, mutated in epidermal growth factor receptor tyrosine kinase domain).

culture (Figure 1B). Treatment efficacy was found to be positively correlated with TTFields intensities, as previously described (data not shown).[6,7] Together, these in vitro findings support our previous observation, which suggest a cell type–specific optimal effective frequency for TTFields therapy.

TTFields Have an Additive Inhibitory Effect on NSCLC Cell Proliferation When Combined with Chemotherapeutic Agents In Vitro

Adjuvant TTFields have previously been shown to increase the sensitivity of cancer cells to various chemotherapeutic agents.[9] To assess whether adding TTFields to standard lung cancer chemotherapy affects the responsiveness of NSCLC cells, we treated the cell lines with TTFields (1.75 V/cm RMS, 150 kHz) combined with pemetrexed (Figure 2A), cisplatin (Figure 2B), and paclitaxel (Figure 2C) at various concentrations. The antifolate pemetrexed has demonstrated efficacy when used as a single agent in second-line treatment of advanced non-SCC NSCLC,[13] in combination with cisplatin for first-line therapy of advanced non-SCC NSCLC,[14] and as maintenance for this indication in patients whose disease has not progressed after platinum-based first-line chemotherapy.[15] We investigated the effects of combining TTFields with pemetrexed in various NSCLC cell lines. The results (Figure 2A) showed that adding TTFields to pemetrexed led to a decrease in the number of viable cells for all tested concentrations in both H1299 and LLC1 cultures. These

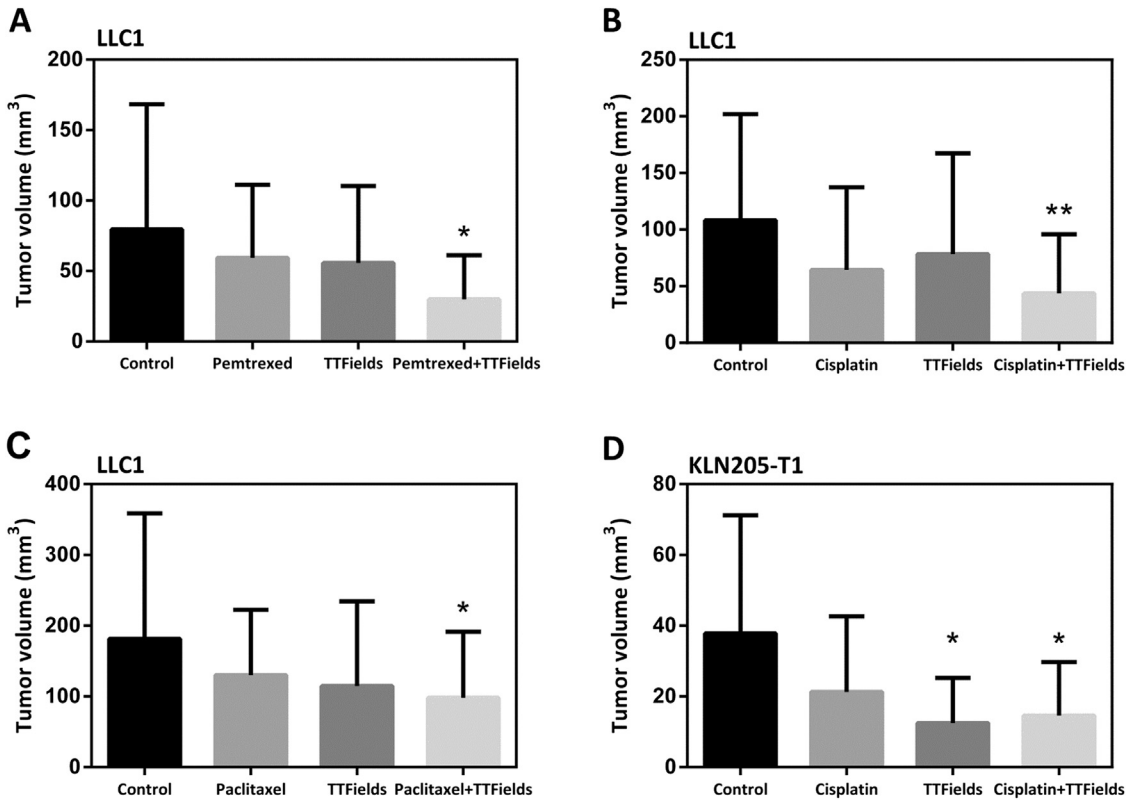

Figure 3. In vivo effects of treatment combination of TTFields therapy and chemotherapy. Volume of LLC1 (Lewis lung carcinoma 1) or KLN205-T1 (murine squamous cell carcinoma cells derived from DBA/2 mice lung tumors) tumors in mice treated with (A) pemetrexed, (B,D) cisplatin, and (C) paclitaxel. *$P < .05$ and **$P < .01$versus control group.

results suggest an additive effect for combining treatments. This effect is possibly explained by the different stages of mitosis at which the two treatment modalities putatively operate: Pemetrexed inhibits three enzymes responsible for purine and pyrimidine synthesis, thereby acting on the G1 and S phases of the cell cycle,[16] and TTFields therapy predominantly acts on cells in the M phase.[6,7,9] Although a platinum-based regimen is standard treatment for patients with NSCLC, this approach may have reached its plateau in terms of efficacy.[17] The combination of cisplatin, a commonly used first-line NSCLC treatment, and TTFields showed a similar pattern of augmented effect against NSCLC cell viability, including in the SCC cell lines HTB-182 and KLN205 (Figure 2B). TTFields therapy in combination with paclitaxel had the same additive effect and led to a decrease in the number of viable cells in cultures of both adenocarcinoma and SCC cells (Figure 2C). The combined effect of TTFields and paclitaxel may be attributed to their shared cellular targets. TTFields forces act on the dipole moment of tubulin chains, leading to misalignment of mitotic spindle filaments[6] and paclitaxel stabilizes the link between individual tubulin dimers, subsequently increasing the length and average dipole moment of the tubulin filaments. This, in turn, could increase the TTFields-induced forces and sensitize cells to TTFields. Interestingly, the KLN205 cell line, though resisting high-concentration paclitaxel (inhibitory concentration 50 [IC_{50}] >100 nmol/L) (data not shown), was sensitive to TTFields (Figures 1A, 2B), suggesting TTFields also may be effective against cells resistant to paclitaxel. NSCLC with activating EGFR mutations is highly responsive to EGFR tyrosine kinase inhibitors, including erlotinib, an approved agent for the treatment of locally advanced or metastatic NSCLC. Compared with exposure to erlotinib alone, the combination of TTFields and erlotinib led to a significant reduction in HCC827 (EGFR mutant) cell viability (Figure 2D). Overall, these results suggest that adding TTFields therapy to systemic therapies including pemetrexed, cisplatin, paclitaxel, and erlotinib may offer a viable approach for improving clinical outcomes. Prospective studies to investigate this hypothesis are warranted.

TTFields Inhibit Tumor Growth in Autologous NSCLC Mouse Models and Can Improve Treatment Efficacy When Combined with Chemotherapy

We orthotopically implanted 6×10^3 LLC1 cells into the left lung of C57BL/6 mice and 5×10^4

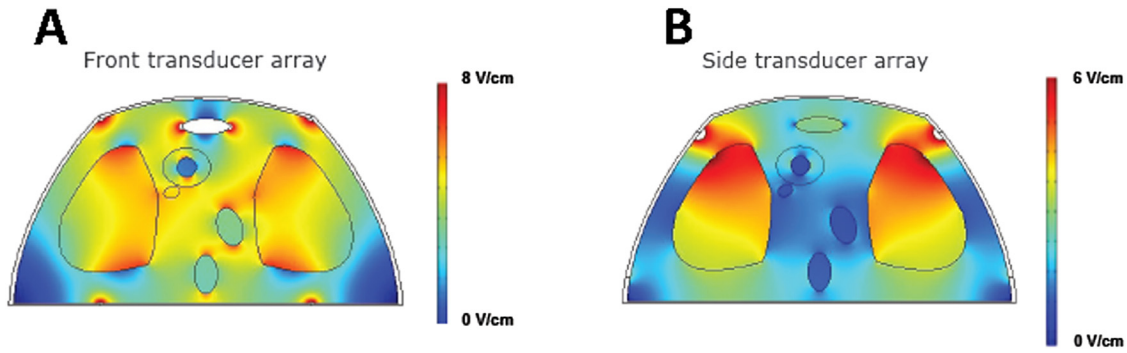

Figure 4. Distribution of TTFields within lungs. Finite-element mesh (three-dimensional mesh) simulations of distribution of TTFields intensity within human chest model demonstrated effective distribution of fields applied by transducer arrays with use of (A) anteroposterior layout (front transducer array) and (B) bilaterally (side transducer array).

KLN205-T1 cells into the left lung of DBA/2 mice. The results (Figures 3A–C) showed that LLC1 tumors exposed to TTFields (1.86±0.67 V/cm RMS) in combination with chemotherapy were smaller than tumors treated with chemotherapy alone. Similar results were observed for KLN205-T1 tumors (Figure 3D). Interestingly, a significant decrease in tumor volume was not observed in mice treated with TTFields with average intensities below V/cm RMS, demonstrating that TTFields are ineffective below a certain threshold. Of note, the improved antitumor activity of the combination of TTFields and chemotherapy also was apparent in autologous tumor studies. These results are supportive of the in vitro findings that suggest combining TTFields therapy with chemotherapy may enhance treatment efficacy. The combination may lead to an increased response and also potentially may permit the use of a lower dose of chemotherapy, thereby reducing systemic toxicity.

Effective-Intensity TTFields Can Be Noninvasively Applied to the Lung Parenchyma

To calculate the TTFields intensity required to effectively alter treatment outcomes in larger organisms, we performed two types of studies: finite-element mesh (FEM) simulations of the electric field distribution in the human chest cavity and in vivo measurements of TTFields intensity within the lungs, liver, and mediastinum of pigs. Intensity and distribution of TTFields within the lung and mediastinum were calculated using FEM simulations of the human chest treated with transducer arrays positioned anteroposteriorly (Figure 4A) or bilaterally (Figure 4B). Despite the spread of the field in the large nonuniform volume, the simulations demonstrated that TTFields of sufficient intensity (2–4 V/cm RMS) can be generated throughout the lungs and mediastinum by applying 150-kHz alternating fields through noninvasive transducer arrays. The simulations also demonstrated protection of hematopoietic tissue through strong attenuation of TTFields by vertebral and costal bony elements. Field intensity was more than 10-fold lower in these regions than in the lung parenchyma. In addition, field intensity was 30% higher in the mediastinum than in the lung parenchyma. In vivo field measurements were performed in the lungs, mediastinum, and liver of anesthetized pigs to validate these simulations. The measurements showed significant correlation with the simulations, indicating that TTFields intensities can be effectively generated within the lungs, liver, and mediastinum (data not shown). Together, our results suggest TTFields therapy can be effectively delivered locally for the treatment of NSCLC.

In summary, the body of evidence from in vitro studies, in vivo studies, and an early phase I/II clinical trial in patients with advanced NSCLC, suggest combining TTFields therapy with systemic chemotherapy may be an approach that offers enhanced efficacy, without incremental toxicity. Further prospective studies to examine the optimal combinations of therapy are warranted.

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