Chapter 6

Enhanced Electric Pulse Technology for the Ablation of Pancreatic Cancer

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Abstract

Electric pulse based technology has been developed and studied as a non-thermal ablation method for local control of pancreatic cancer. Irreversible electroporation (IRE) has shown a significant survival benefit for local advanced pancreatic cancer in clinical trials. However, incomplete ablation with local recurrence and major complications limit the potential of this new technology. We have developed an integrated moderate heating electric pulse delivery system which consists of controllable tumor heating, multi-parameter monitoring and electric pulse delivery. The impedance of tumor is greatly decreased after moderate heating at 42°C for 1–2 min, which does not cause any cell death. Moderate heating significantly enlarges the ablation zone of tumor treated with IRE. In contrast to IRE alone, moderate heating assisted IRE results in a high rate of complete tumor regression and a significant longer median survival. Another electric pulse technology, nanosecond electric pulses, has been assessed for the treatment of pancreatic cancer as well. Nanosecond electric pulse treatment achieves more survival benefit in animals with partial tumor ablation than those treated with IRE and leads to a vaccine-like protective effect in animals with complete local ablation. More studies are needed to demonstrate the advantages and translational feasibility of the enhanced electric pulse technologies.

Keywords: electric pulses, tumor ablation, pancreatic cancer, irreversible electroporation, moderate heating, nanosecond electric pulses

1. Introduction

The incidence of pancreatic cancer is relatively low. It only counts for 2% of all cancers [1, 2]. However, pancreatic cancer is a serious global health issue due to its extremely high mortality

and poor prognosis. The overall 5-year survival rate is 5–8% [2–4]. In contrast to other major cancers with decreasing mortality rates, the mortality rate of pancreatic cancer has been gradually increasing in the past 50 years [2]. It is predicted to be the second leading cause of cancer-related deaths in the United States and Europe by 2030 [5]. There are several reasons for the dismal outcome of pancreatic cancer. There are no early signs and symptoms for pancreatic cancer. There is no screening tests or early diagnostic methods. Pancreatic cancer is often diagnosed at a late stage with a large tumor burden. It is notoriously resistant to chemotherapy and radiotherapy [6–8]. Surgery is the only way to potentially achieve complete cure of early stage pancreatic cancer, which counts for approximately 15–20% of the patients [9]. Nevertheless, the 5-year survival rates of surgical resection are only 13.6–17.5% [10]. The poor prognosis after surgery is due to a high incidence of local recurrence and distant metastases [11, 12].

In the last decade, much effort has been made to develop ablative technologies for local pancreatic tumor control and the improvement of quality of life and survival. An electric pulse technology, irreversible electroporation (IRE) as a non-thermal ablation method has been investigated in animal models for tumor ablation [13, 14]. Recently IRE has been studied in clinical trials for liver [15], renal [16], prostate [17] and pancreatic cancers [18–21]. Martin et al. reported that overall survival increased 6–8 months in patients with local advanced pancreatic adenocarcinoma treated with IRE [22]. A systematic review demonstrated significant survival benefits with reducing the risk of injury to vessels and ducts after treating advanced pancreatic cancer with IRE [23]. IRE has been demonstrated some advantages in contrast to thermal ablation technologies, which are associated with high morbidity and mortality due to thermal damage to adjacent structures. However, local recurrence [22, 24–26] and various rates of major complications [18, 22, 25] are two major issues that restrict the benefit of IRE treatment. Thus, the enhancement technologies for IRE or novel non-thermal electric pulse technologies, which can increase complete tumor ablation and/or decrease adverse effects, are needed to further improve the quality of life and long term survival.

Here we introduce two promising electric pulse technologies, moderate heating (MH) enhanced IRE and nanosecond electric pulses (nsEPs) for the treatment of pancreatic cancer, and present our preclinical findings demonstrating their potential advantages in contrast to current IRE technology.

2. Moderate heating enhances the therapeutic efficacy of irreversible electroporation for pancreatic cancer

2.1. Background

The impedance change of biological tissues at various temperatures has been investigated for over three decades [27, 28]. A decrease of impedance means the increase of tissue conductivity, which is equal to an elevated current and a large electrical energy delivery to the tissue or tumor under a certain electric field. We found that tumor ablation zone could be significantly

enlarged when preheating with moderate temperature increase was applied. This result led to our hypothesis that a moderate increase in the temperature of the target tumor could decrease tumor impedance, thereby sensitizing the target tumor for IRE tumor ablation. To test this hypothesis, we first developed a controllable tumor heating unit and an impedance monitoring unit, then integrated these two units into an electric pulse supplier. In additional to treating tumor with IRE, this integrated electric pulse delivery system has the capacity to heat the targeting tumor, maintain at a set temperature and monitor impedance changes of the treated tissue in real time.

2.2. Experimental design

Ex vivo IRE tumor ablation was assessed in a 3D agarose cell culture model, which was described in the literature [29]. Pan02 mouse pancreatic cancer cells were used to make the 3D tumor model. The IRE parameters were pulse duration 100 μs, frequency 1 Hz, 80 pulses, and electric fields 750 V/cm. The four-needle electrode was utilized to deliver this electric pulse protocol. A thermopile was integrated into the electrode for a real-time temperature monitoring and a fiber optic laser located at the center of the electrode was used for tumor heating. After IRE treatment, tumor was stained with propidium iodide (PI, 4 μg/ml) for 30 minutes. Images were taken using a Leica MZFLIII fluorescence stereomicroscope equipped with a Leica DFC420 C CCD camera. Cell death or ablation zone was quantified with ImageJ software (imagej.nih.gov/ij/).

A syngeneic mouse Pan02 pancreatic cancer model was established for the evaluation of this moderate heating enhanced IRE (MHIRE) system. Female C57BL/6 mice (6–8 weeks of age) were injected with 1×10^6 Pan02 cells in 50 μL Dulbecco's phosphate buffered saline on the left flank. The size of primary tumor was assessed by digital calipers twice a week. Tumor volume was determined as described in the literature [30]. Tumors were treated when it reached 8–10 mm in diameter with an average tumor volume of 250–300 mm^3. The IRE parameters were pulse duration 100 μs, frequency 1 Hz, 90–120 pulses, and electric fields 1500–2500 V/cm. A four-needle electrode arranged in an array of 7×5 mm spacing was used to treat pancreatic tumor.

MH was defined at 42°C within 2 min, which is below the threshold of pain sensation [31] and does not cause cell death [32]. A calibration of MH protocol was done prior to the MHIRE treatment. A thin thermocouple was inserted in the bottom part of tumor and the time it took the reading to reach 42°C was recorded. It took 20–60 s for the internal tumor temperature to reach 42°C when the surface target temperature was set to 45°C by laser heating. So, based on the calibration results, we decided that pre-heating tumor for 60 s would allow the inside of the tumor to be at the correct internal temperature before IRE was performed.

2.3. Results and discussion

2.3.1. Moderate heating decreased the impedance of tumor

The change of impedance during the IRE or MHIRE treatment is shown is **Figure 1**. The baseline impedance of each tumor was different; however, preheating tumor with a moderate

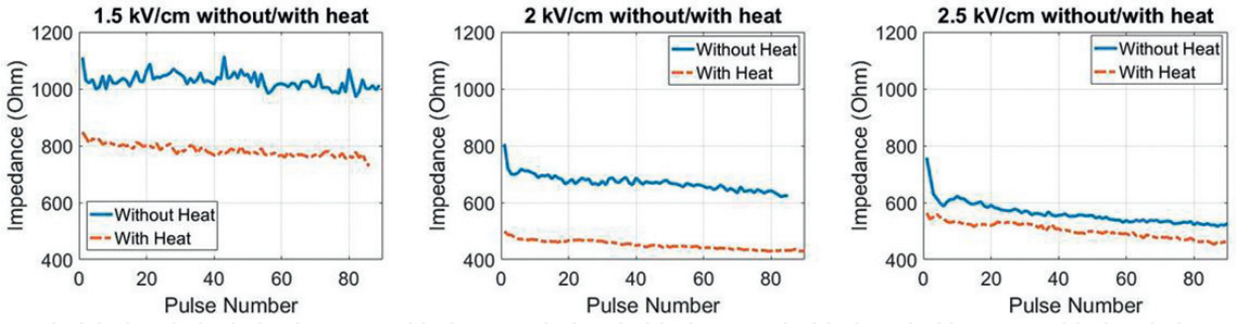

Figure 1. Tumor impedance change during IRE or MHIRE treatment. Each data point represents an average impedance reading of 4–5 tumors. IRE parameters: 100 μs pulse width, 90 pulses, frequency of 1 Hz and applied electric fields of 1.5 kV/cm (left), 2 kV/cm (middle) and 2.5 kV/cm (right).

temperature increase could reduce all of them by 200–300 Ω or 15–38% of the baseline impedance, which occurred prior to the IRE treatment. Consistent to the other group's report [33], IRE could result in the decease of tumor impedance as well. It appears that the reduction of the impedance was associated with the strength of the electric field. The higher electric field, the more the impedance was reduced by the end of the treatment. Additionally, MH also reduced the fluctuation of impedance changes, which may indicate that MH improves homogeneity of the tumor physical property. The average drop of impedance was 39.1 to 46.6% for MHIRE with 2000 to 2500 V/cm and 22.4 to 30.5% for IRE with the same electric field.

The impedance decrease of tumor was likely correlated to the complete tumor ablation of IRE [33]. Given an approximate 40% decrease of tumor impedance, theoretically IRE at 2500 V/cm should be equivalent to MHIRE at 1500 V/cm. It means MHIRE could reduce the electric field of IRE and achieve the same level of efficacy for tumor ablation. Interestingly, MH was observed to decrease the impedance fluctuation of the tumor as well. Tumor is not a homogenous structure but with multiple types of cells and extracellular matrix [34, 35]. A heterogeneous impedance map of tumor tissue [36] is expected and may contribute to the incomplete ablation of IRE. This feature of MH might also contribute to more complete tumor ablation.

2.3.2. Controllable MH enlarged ex vivo *tumor ablation and enhanced the therapeutic efficacy of IRE for pancreatic cancer*

More cell death was observed after Pan02 tumor cells in a 3D agarose gel were treated with MHIRE with a four-needle electrode, while tumor cells treated with MH alone did not result in any cell death (**Figure 2**). The ablation zone or total cell death increased 1.4-fold with MHIRE at an electric field of 750 V/cm ($p < 0.05$) comparing to those treated with IRE at the same electric field.

Tumor bearing animals were treated with either IRE or MHIRE at 1500 V/cm. The IRE treatments alone had no significant influence on the tumor growth. However, a synergistic effect was seen in the IRE treatment when the tumor was preheated to 42°C (**Figure 3**). Tumors treated with MHIRE were all significantly smaller than those in the control group or those in IRE-alone group on post-treatment days 4, 7, 11, 13 and 14. However, no long-term complete tumor regression was obtained under either IRE or MHIRE protocols. In order to obtain

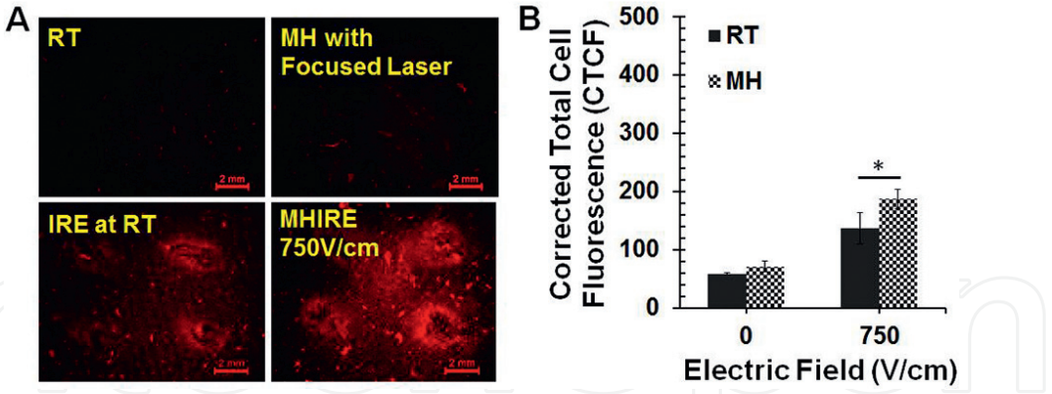

Figure 2. Enlargement of *ex vivo* tumor ablation zone with MHIRE. A 3D agarose gel Pan02 tumor model was treated by IRE or MHIRE. Area with red color was zone of dead cells indicated by propidium iodide (PI) staining. RT: room temperature; MH: samples preheated with laser. Corrected total cell fluorescence (CTCF) was analyzed by ImageJ software. n = 3–4. *: $p < 0.05$ (t-test).

complete tumor regression, IRE or MHIRE with elevated electric fields were adopted to treat tumor. At the electric field of 2000–2500 V/cm, MHIRE significantly prolonged median survival by roughly two times with 84 days in contrast to the control mice ($p < 0.001$) (**Figure 4**). Despite the higher electric fields, IRE treatment alone could not achieve long-term complete tumor regression. It only extended medial survival for 3 days. Median survival was 43 days for the control tumor animals and 46 days for the IRE treated animals. More importantly, 55.6% (5/9) of the tumor-bearing animals treated with MHIRE were long term tumor-free.

It was noticed that IRE alone was unable to achieve complete tumor ablation in this mouse Pan02 tumor model though the IRE protocol was similar to those used in other animal studies and clinical trials. Jose et al. reported that IRE treatment resulted in 25% of complete tumor ablation [37]. In that study, a comparable IRE protocol (100 μs, 1 Hz, 2500 V/cm and pulse number 90) was used to treat xenograft human BxPC-3-luc pancreatic tumors in athymic nude mice. Local recurrence was reported relatively low in clinical trials, 11% by Kluger's group [25] or 27.8% by Martin's group [22]. Though multiple factors including the tissue type of tumor, its size, the IRE protocol and electrode configuration could contribute to the incomplete ablation, the physical properties of the target tumors especially to the impedance likely play a critical role. The 750–800 Ω impedance of mouse Pan02 tumor (**Figure 1**) is much higher than the 100–120 Ω impedance of human pancreatic cancer reported by Dr. Martin's group [33]. Such a big difference of impedance may explain why the mouse Pan02 tumor is difficult to be successfully ablated by the IRE treatment.

Though MH alone did not result in any cell death (**Figure 2**) and had no impact on tumor regression and animal survival (**Figure 3**), it was demonstrated to synergize with IRE on tumor ablation zone *in vitro* (**Figure 2**), to diminish tumor growth (**Figure 3**) and to improve long-term survival *in vivo* (**Figure 4**). Together with the impedance changes of tumors (**Figure 1**), we have validated a novel technology and concept that the therapeutic efficacy of IRE can be enhanced by MH with a consequent decrease of tumor impedance. Is it feasible to translate this technology into an effective therapy for pancreatic cancer? The MHIRE system developed in this study has been utilized to successfully treat tumor with relative small size (less than 1 cm).

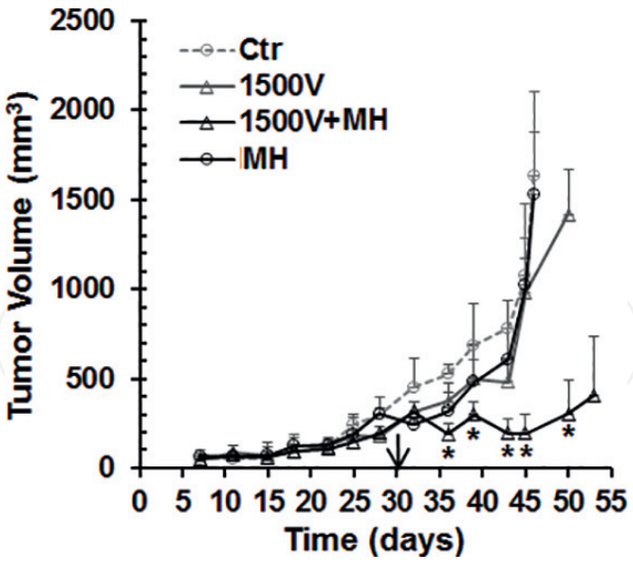

Figure 3. Pancreatic tumor growth after IRE or MHIRE treatment. Pan02 pancreatic tumors with the size of 8–10 mm were treated with IRE or MHIRE at day 31 indicated by black arrow. IRE parameters: pulse duration 100 μs, frequency 1 Hz, pulse number 90 and applied electric fields 1500 V/cm. Ctr: no treatment (n = 4); MH: tumor heated with laser at 42°C for 2 min; 1500 V: IRE at 1500 V/cm (n = 4 mice); 1500 V + MH: Tumor preheated with laser at 42°C with IRE at 1500 V/cm (n = 8). *: $p < 0.05$, or $p < 0.01$ or $p < 0.001$ for MHIRE vs. IRE or Ctr (one way ANOVA).

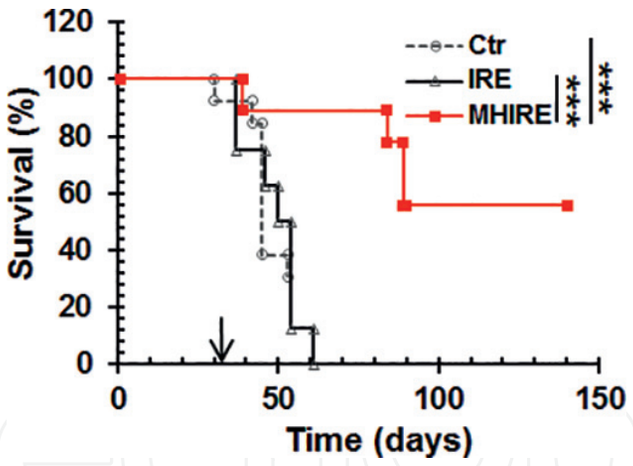

Figure 4. Kaplan-Meier survival curves of mice treated with IRE or MHIRE. Pan02 pancreatic tumors with the size of 8–10 mm were treated with IRE or MHIRE at day 31 indicated by arrow. IRE parameters: pulse duration 100 μs, frequency 1 Hz, pulse number 90 and applied electric fields 2000-2500 V/cm. Ctr: No treatment (n = 8 mice per treatment group); IRE: Treated with IRE (n = 8); MHIRE: Tumor preheated with laser at 42°C with IRE (n = 9). ***: $p < 0.001$ (LogRank test).

However, as is known, most tumors in patients are larger than 1 cm, especially for later stage cancers, which are the targets of the IRE treatment. This limitation of treatable size can be addressed with the adjustment of the electrode configuration, to cover a larger area. Meanwhile, the depth of laser heating and its thermal distribution needs to be profiled, and the refined MHIRE system will be calibrated/reprogrammed and optimized in an *in vivo* pancreatic cancer

model with tumor size relevant to the clinical settings. The extension of heating area can be resolved by the integration of multiple infrared laser beams and/or additional optic lens, increase of needle gap and length. To heat large and deep tumors, different laser sources [38] can be adopted. Moreover, other heating methods, including focused ultrasound [39], microwave [40] or radiofrequency [41], could be employed for the purpose of MH.

3. Nanosecond electric pulses for the treatment of pancreatic cancer

3.1. Background

An electrical engineering technology, nanosecond electric pulses (nsEPs), has been developed and studied by Dr. Schoenbach's [42] and other groups [43]. NsEPs are assumed non-thermal if the appropriate parameters especially the low frequencies are selected. Similar to IRE, nsEPs have been utilized to treat cancer in animal models for local tumor ablation [44–46]. Beyond the local tumor ablation, a vaccine-like protective effect has been observed by two groups [44, 47]. The vaccine like-protection effect has been demonstrated by our group [48] in a poorly immunogenic breast cancer model as well. We have demonstrated that local nsEP tumor ablation elicits an anti-tumor immunity to prevent distant metastases, reject established distant tumors and protect animals from secondary tumor challenge. Thus, nsEP therapy shows additional advantages, in addition to local tumor eradication.

NsEPs have been reported for the treatment of pancreatic cancer in two studies [36, 49]. However, whether immune protection is induced by the nsEP treatment is unknown because xenograft tumors in immune deficient animals have been used in both studies. To assess if nsEP ablation could induce antitumor immunity and achieve additional benefits beyond local ablation for pancreatic cancer, a syngeneic mouse Pan02 pancreatic cancer model was utilized in this study.

3.2. Experimental design

A syngeneic mouse Pan02 pancreatic cancer model was established as above mentioned. Tumors were treated when it reached 5–7 mm or 8–10 mm in diameter with an average tumor volume of 40–120 mm^3 (small) or 250–300 mm^3 (large). The nsEP parameters were pulse duration 100 or 200 ns, frequency 1–3 Hz, pulse number 600–1200, and electric fields 30–50 kV/cm. Pancreatic tumors were treated with either a four-needle electrode with gaps of 5 × 7 mm or a pitch electrode, which was selected from three configurations including 2 mm gap with 6 mm in diameter, 3 mm gap with 8 mm in diameter and 4 mm gap with 10 mm in diameter. In comparison, pancreatic tumors were also treated with IRE. The IRE parameters and the choice of electrode were described in the previous section.

To assess if a vaccine-like protection occurred after pancreatic cancer was treated with nsEPs, tumor free mice were challenged with 0.5 million live Pan02 tumor cells on the right flank. Tumor growth was monitored as above mentioned.

3.3. Results and discussion

3.3.1. NsEP treatment resulted in complete tumor regression or extension of survival for animals with incomplete tumor regression

As shown in **Figure 5**, a single nsEP treatment achieved 50–100% complete tumor regression dependent on the doses of nsEPs. In contrast to the IRE treatment, muscle contraction was greatly reduced with the nsEP treatment. Both pitch electrode and two-plate suction electrodes were safe and no mortality was found. A minor issue was that scab was formed after the nsEP treatment. It usually shed within 2–3 weeks and left a small scar or no visual changes on the skin.

Extension of survival was achieved even with partial tumor ablation regardless of whether pancreatic cancer with small size (5–7 mm) or big size (8–10 mm) was treated, and median survival was extended to 63 days (**Figure 6A**) if small tumors were treated, or to 68 days (**Figure 6B**) if large tumors were treated, in contrast to 45 days for the control animals. However, the survival benefit was only present in large tumors treated with IRE but not in small tumors if the tumors were partially ablated. Median survival was extended to 50 days if large tumor was treated, in contrast to 45 days for the control animals (**Figure 6B**). Actually, the median survival was shortened to 40 days if the small tumors were not completely ablated with IRE. Obviously, tumor growth was accelerated and lost heterogeneous pattern after partial IRE ablation (**Figure 7**). The same phenomenon was reported in literature and explained as cancer stem cell activation [50]. Nevertheless, this was not seen in the animals treated with nsEPs. It suggests different cell death mechanisms or possible inhibition of immune responses may occur.

3.3.2. A vaccine-like protective effect was resulted from the nsEP treatment

As shown in **Figure 8**, tumor free mice after the nsEP treatment were able to impede or prevent the growth of challenging tumors. Noticeably, there was a significant difference between the two nsEP protocols. Majority of tumor free mice (66.7%) after the 100 nsEP treatment were successfully protected from the second live tumor challenge whereas no protection but only growth inhibition of tumor was observed in animals treated with the 200 nsEPs. Nevertheless, neither protection nor growth inhibition was seen in the animals treated with IRE.

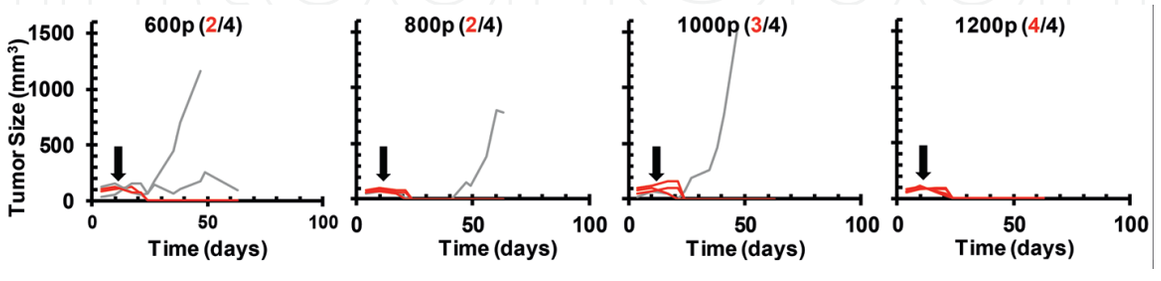

Figure 5. Pancreatic tumor growth after the nsEP treatment. Pan02 pancreatic tumors with the size of 5–7 mm were treated with nsEPs at day 11 indicated by black arrow. nsEP parameters: 200 ns, 2 Hz, 30 kV/cm, and pulse numbers 600, 800, 1000 or 1200, indicated by 600p, 800p, 1000p or 1200p, separately. Number of tumor free mice vs. total number of treated mice was indicated.

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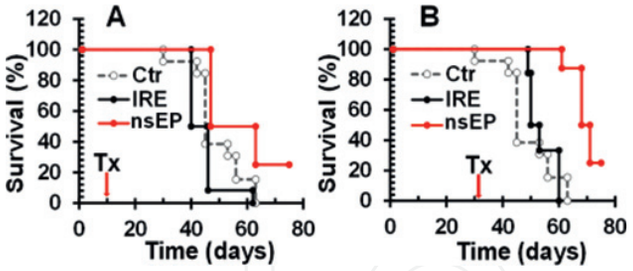

Figure 6. Survival extension in animals with incomplete tumor regression after the nsEP treatment. Pan02 pancreatic cancer was treated with IRE or nsEP. Only animals with incomplete tumor regression were included here. **A,** tumor size with 5–7 mm was treated (n = 13, 12 or 4 for Ctr, IRE or nsEP). Tx: Treatment at day 7 (IRE) or 11 (nsEP). **B,** tumor size with 8–10 mm was treated. Ctr: No treatment; IRE: Treatment with IRE; nsEP: Treatment with 200 ns, 2 Hz, 30 kV/cm and 600–1200 pulses (n = 13, 6 or 7 for Ctr, IRE or nsEP). Tx: treatment at day 31.

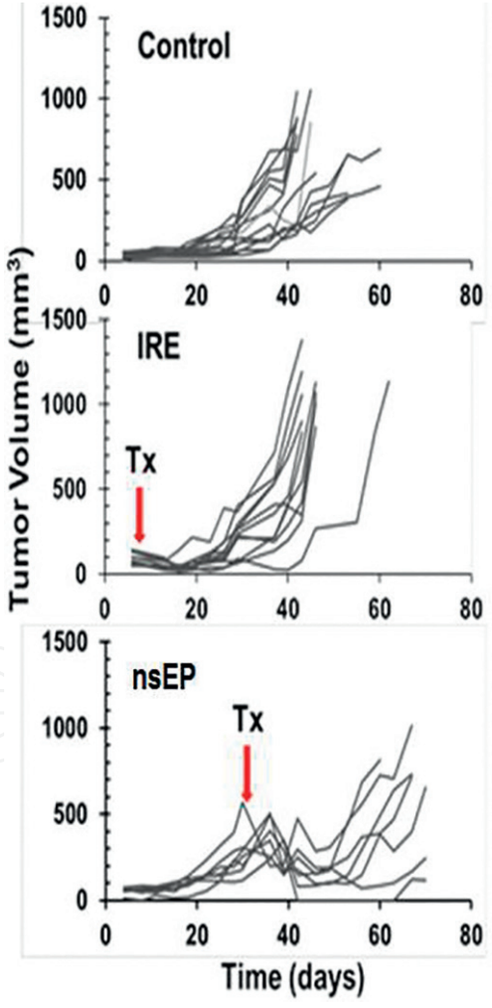

Figure 7. Pancreatic cancer growth after treatment with IRE or nsEP*. Control (n = 13): no treatment. IRE (n = 12): treated with IRE. Tx: treatment day 7. nsEP (n = 7): treated with nsEP (200 ns, 30 kV/cm, 2 Hz with 800–1000 pulses), Tx: treatment day 31. *: Only animals with partial tumor regression were included to assess the effect of treatment on tumor regrowth.

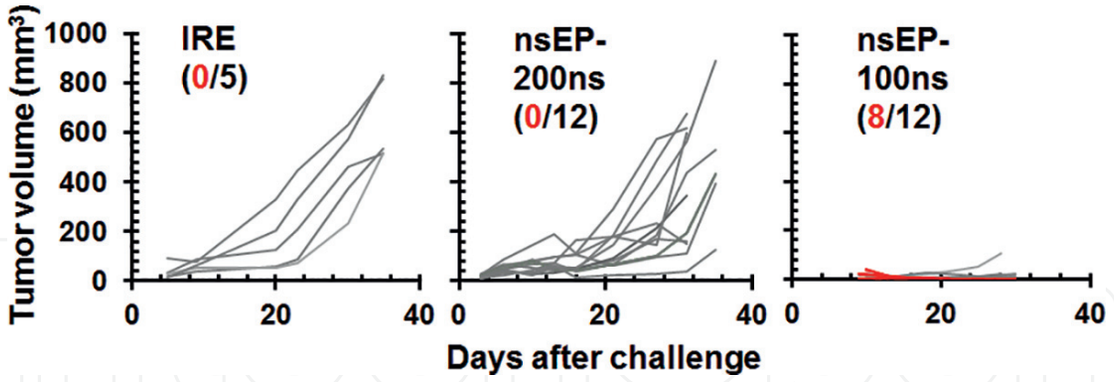

Figure 8. A vaccine-like protection effect after the nsEP treatment. Growth curves of second challenge pancreatic tumors in tumor-free animals after IRE or nsEPs. Primary pancreatic tumors were treated with IRE (**IRE**), nsEPs with 200 ns, 2 Hz, 30 kV/cm and 600 -1200 pulses (**nsEP-200 ns**), or nsEPs with 100 ns, 2 Hz, 50 kV/cm and 800 -1200 pulses (**nsEP-100 ns**). Number of protective mice vs. total number of challenged mice was indicated. $p < 0.05$ for nsEP-200 ns vs. IRE and $p = 0.001$ for nsEP-200 ns vs. nsEP-100 ns (Chi Square test).

Surprisingly, the protective rates between two sets of nsEP parameters are very different. A high rate of protection from the second live tumor challenge, 100%, has been observed in both mouse breast cancer [48] and rat hepatocellular cancer models [44] after the same 100 nsEP treatment. Does this mean 100 nsEPs are more favorable to induce immune protection than 200 nsEPs? The answer is not clear because 100 nsEPs has eradicated local mouse lung squamous cell cancer (KLN205) but has failed to result in any vaccine-like protection (0/19 protection in our unpublished data). It's very likely that cancer cell types and distinctive tumor microenvironments play a critical role on the induction of immunity following the nsEP tumor ablation.

The growth inhibition of local recurring tumors and the second challenging tumors suggests that underlying common immune responses are induced after the nsEP treatment. It's critical to understand the mechanisms causing the differential responses and outcomes between IRE and nsEPs or among various nsEP parameters, so it is possible for researchers to design more effective therapeutic strategies, such as further optimization of the system or a combination therapy with other immunomodulators. Currently, we are investigating cell death mechanisms, local and systemic immune responses, and the changes of tumor microenvironments following the nsEP tumor ablation.

4. Conclusion

Two electric pulse-based technologies have been studied to treat pancreatic cancer in a syngeneic mouse pancreatic cancer model. A novel MHIRE system has been developed. This MHIRE system has three functions including controllable tumor heating, impedance monitoring and electric pulse delivery. MH has been demonstrated to decrease the impedance of tumor, to enlarge the tumor ablation zone of IRE *ex vivo* and to enhance the complete tumor ablation of the IRE treatment *in vivo*. The MHIRE treatment significantly improves the therapeutic efficacy of the IRE treatment. In contrast to the IRE treatment, nsEP tumor ablation showed distinctive outcomes and potential advantages. If partial ablation occurred after either the IRE or the nsEP treatment, animals treated with nsEPs received survival benefit. If complete local ablation was achieved, animals treated with nsEPs but not with IRE were able to reject secondary tumor

challenge or to diminish its growth. An induction of antitumor immunity following the nsEP treatment is highly suggested to account for this vaccine-like protective effect. For both MHIRE and nsEPs for the treatment of pancreatic cancer, our data are preliminary and more studies are needed to further optimize these technologies, elucidate the underlying mechanisms and evaluate their translational feasibility.

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Conflict of interest

R. Heller and S.J. Beebe own stock in Pulse Biosciences, Inc.

All other authors declared no potential conflicts of interest.

Notes

The data present in the second section have been published in or modified from the journal of Scientific Report (see [27]).

Author contributions

S.G. conceived, designed, and supervised the studies. S.G., K.S., R.H, and C.J. designed and developed the MHIRE system. N.B., C.E., J.H. S.B., and S.G. conducted the experiments. S.G. analyzed and interpreted the data. All authors contributed to writing, editing, and review of the manuscript.

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