TITLE:

A Syngeneic Pancreatic Cancer Mouse Model to Study the Effects of Irreversible Electroporation

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SUMMARY:

Irreversible electroporation (IRE) is a non-thermal ablation technique used for the treatment of locally advanced pancreatic cancer. Being a relatively new technique, the effects of IRE on the tumor growth are poorly understood. We have developed a syngeneic mouse model that facilitates studying the effects of IRE on pancreatic cancer.

ABSTRACT:

Pancreatic cancer (PC), a disease which kills approximately 40,000 patients each year in the US, has successfully evaded several therapeutic approaches including the promising immunotherapeutic strategies. Irreversible electroporation (IRE) is a non-thermal ablation technique that induces tumor cell death without destruction of adjacent collagenous structures, thus enabling the procedure to be performed in tumors very close to blood vessels. Unlike thermal ablation techniques, IRE results in gradual apoptotic cell death, along with immediate ablation induced necrosis, and is currently in clinical use for selected patients with locally advanced PC. An ablative, non-target specific procedure like IRE can induce a myriad of responses in the tumor microenvironment. A few studies have addressed the effects of IRE on tumor growth in other tumor types, but none have focused on PC. We have developed a syngeneic mouse model of PC in which subcutaneous (SQ) and orthotopic tumors can be successfully treated with IRE in a highly controlled setting, facilitating various longitudinal studies post procedure. This animal model serves as a robust system to study the effects of IRE and ways to improve the clinical efficacy of IRE.

INTRODUCTION:

Pancreatic ductal adenocarcinoma (PC) is projected to become the second leading cause of cancer deaths in the US around 2020[1]. The vast majority of patients diagnosed with PC will eventually die from distant metastatic disease[2]. The PC microenvironment is notoriously immunosuppressive and chemoresistant. Its desmoplastic stroma contains a scarcity of effector (anti-tumor) T cells and a prominence of immunosuppressive leukocytes, including tumor-associated macrophages (TAMs), myeloid derived suppressor cells (MDSCs), and regulatory T cells (Tregs)[3]. These underlie the need to develop multimodal strategies that counteract these effects of the microenvironment.

IRE has been developed as a non-thermal method of tumor ablation. Unlike thermal ablation techniques, IRE does not cause rapid coagulative necrosis but instead results in gradual apoptotic cell death[4]. Importantly for pancreatic tumors, IRE is not vulnerable to "heat sink" effects and can be performed right next to blood vessels[5]. This technology has 510(k) clearance from the FDA[6] and is currently being used clinically, for selected patients with locally advanced or borderline resectable pancreatic cancer. In the largest published series of IRE for PC[7], the median survival of patients undergoing IRE was approximately double the survival of patients treated with modern chemotherapy alone without resection[8,9].

Several studies have demonstrated that thermal ablation induces a systemic immune response in other tumor types (reviewed in Chu et al.[10]). Radiofrequency ablation (RFA) in animal tumor models leads to increased T cell infiltrates[11,12], including an increase in activated natural killer (NK) cells in hepatocellular cancer patients[13,14], and a decrease in immunosuppressive Tregs in lung cancer patients[15]. A much smaller number of studies have examined immune, microenvironmental, and injury responses to IRE[16]. IRE has been shown to stimulate a systemic immune response in immunocompetent mouse models in which the growth of secondary (contralateral) renal cell allografts was reduced or prevented by IRE of a primary tumor two weeks earlier[17]. They also observed that immunocompetent mice required less voltage for complete regression than did immunocompromised mice. It has been hypothesized that IRE may result in improved antigen presentation compared to the coagulative necrosis of thermal ablation, but this has not been specifically studied.

We have developed a syngeneic mouse model of PC from the KPC-Luc 4580 cell line (gift from J.J. Yeh at University of North Carolina), which was derived from a tumor that developed in a male LSL-Kras$^{G12D/+}$; LSL-Trp53$^{R172H/+}$; PDX1$^{Cre/+}$; LSL-ROSA26 $^{Luc/+}$ mouse, to study the local and systemic effects of IRE[18,19]. This luciferase-expressing cell line is immunogenic and also tumorigenic in immunocompetent C57BL/6 mice when injected SQ or orthotopically and reliably produces liver metastases when injected into the spleen. We have utilized a programmable square wave pulse generator to deliver 100-µs pulses of electricity at a voltage/distance ratio of 1,500 V/cm using a two-needle array probe (separated by 5 mm) or platinum tweezer-trodes to SQ or orthotopic tumors, respectively, in mice to model the effects of IRE in a small animal.

PROTOCOL:

All animal experiments performed following this protocol must be approved by the respective Institutional Animal Care and Use Committee (IACUC). All procedures described here have been approved by IACUC UCSD.

1. Procure recipient animals

NOTE: The KPC-Luc 4580 cell line was established from a tumor arising in a LSL-Kras$^{G12D/+}$; LSL-Trp53$^{R172H/+}$; PDX1$^{Cre/+}$; LSL-ROSA26 $^{Luc/+}$ mouse (KPC), which is a genetically engineered model of PC on a C57BL/6 background. The advantage of this cell line is that it constitutively expresses luciferase-enabling tumor monitoring in orthotopic models. However, other cell lines may also be used as long as they are compatible with the genetic background of the recipient mice.

1.1. Determine the experimental groups and the number of animals per group in advance.

1.2. Choose the age (6–10-week old mice recommended) and sex of the animals being used according to the hypothesis and the cell line used.

2. Culture cells

2.1. Use Dulbecco's modified eagle medium (DMEM): nutrient mixture F12 (50:50) media containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotic (Complete growth media) to grow KPC-Luc 4580 cells.

2.2. If frozen, thaw the cells 4 days prior to tumor implantation.

2.3. Grow the cells at 37 °C with 5% CO_2 and 95% humidity under aseptic conditions.

2.4. Trypsinize the cells with 0.25% Trypsin for 5 min at 37 °C before they reach > 80% confluency and neutralize the trypsin by adding twice the volume of complete growth media.

NOTE: These cells do not establish tumors efficiently in mice if they are used at 100% confluency or if they are passaged for more than 12 times. Hence, it is advisable to freeze the cells at earlier passages under optimal confluency.

2.5. Count the cells using a hemocytometer and proceed to tumor induction if adequate numbers are present (0.5–1 x 10^6 cells/mouse). If not, expand the cells for one more passage until adequate cell numbers are obtained.

3. Induction of SQ tumors

3.1. Prepare the materials required: P1000 and P100 pipettes and tips, 1 mL syringe, 25 G needles, basement membrane matrix (BMM), DMEM basal media, 1.5 mL centrifuge tubes. Keep all materials sterile and cold (4 °C) until the end of the procedure. Also keep hair clippers and alcohol swabs ready for animal use.

3.2. Trypsinize (as per step 2.4) and count the cells, and resuspend them at a concentration of 2–4 x 10^7 cells/mL of cold DMEM.

3.3. Add an equal volume of 100% BMM to make a final cell suspension in 50% BMM. The final volume should amount to 50 µL of cell suspension for each mouse required. Prepare 10% in excess to accommodate the dead space in the syringe and for any pipetting errors.

3.4. Aliquot the suspension in 550 µL volumes in 1.5 mL centrifuge tubes placed in ice.

3.5. Restrain the animals (6-week old male C57BL/6 mice) using a restrainer or manually.

NOTE: Alternatively, mice can be anaesthetized with 2.5% isoflurane in oxygen gas at a flow rate of 2 L/min using a precision vaporizer.

3.6. Shave the site of injection, which is preferably on a flank above a leg, using a clipper. Using an alcohol swab, clean the site of injection twice.

3.7. Draw 250 µL of cell suspension at a final concentration of 1–2 x 10^7 cells/mL in a 1 mL cold syringe and remove any air bubbles by moving the piston up and down. Attach a 25G needle to the syringe and make sure to fill the space within the needle.

3.8. Carefully insert the needle under the skin at a 30° angle to the body near the flanks, making sure that the needle does not pierce the peritoneal cavity or the musculature of the limbs.

3.9. Flatten the needle parallel to the body, and flatten wrinkles on the skin. Slowly inject 50 µL of cell suspension. Hold the needle at the site of injection for 10 s to allow the BMM to solidify to prevent any leakage.

3.10. Slowly withdraw the needle keeping it parallel without any sideways movement. Gently clean the site of injection with an alcohol swab and release the mouse to its housing.

NOTE: If the mouse was anesthetized during the procedure, monitor the animal until it regains its righting reflexes.

3.11. Monitor the growth of the tumor regularly using calipers until it reaches 5 mm in diameter.

4. Induction of orthotopic tumors

4.1. Prepare the materials required. Autoclave the surgical tools such as scissors, scalpels, needle drivers, and pointed and flat-tipped forceps. Keep handy sterile sutures (absorbable 6-0 and non-absorbable 4-0), alcohol swabs, hair clippers, depilatory cream, eye lubricant, cotton gauze, 10% povidone iodine solution, surgical drapes, heating pads, buprenorphine, and anesthetic agents.

4.2. Euthanize a donor mouse (preferably of the same background required for the orthotopic model) carrying a SQ KPC tumor using a slow fill CO_2 chamber. Excise the SQ tumor carefully under sterile conditions in a BSL-2 hood using sterile forceps and microscissors shortly before orthotopic implantation. During tumor excision take care not to puncture the peritoneal cavity and wash the tumor immediately with 2 exchanges of sterile PBS.

4.3. Using sterile scalpels, mince the excised tumor into 1 mm^3 pieces, place them on a Petri dish containing cold sterile DMEM basal media, and store on ice until implantation.

4.4. Administer the recipient mouse 0.05–1 mg/kg buprenorphine analgesic SQ, 30 min prior to surgery.

4.5. Anesthetize the recipient mice with 2–3% isoflurane in oxygen (2 L/min) using a precision vaporizer (or other anesthetic agents). Keep mice on a 37 °C heating pad for the entirety of the procedure. Apply lubricant to the eyes to prevent desiccation. Test the depth of anesthesia by lack of startling reflex initially, and confirm surgical plane anesthesia by the lack of pedal reflex to a gentle toe pinch. Maintain the anesthesia during the entire surgical procedure.

4.6. Place the mouse on its back and gently turn it to its right side so that the left side of the abdomen is exposed. Remove the abdominal hair of the mouse using depilatory cream and clean with gauze to ensure no free hair enters the abdomen post incision. Prepare the left abdomen for surgery using 3 cycles of 10% povidone iodine followed by alcohol wipes to disinfect the skin.

4.7. Using a sterile scalpel, make a 1.5 cm transverse or oblique incision in the skin, 1-cm to the left of the midline, below the ribcage, slightly medial to the spleen. Then, extend the incision through the abdominal musculature, mirroring the overlying superficial incision.

4.8. Locate the spleen using flat tipped forceps and gently externalize it from the abdominal cavity. Retract the spleen using a sterile cotton applicator, and find the tail of the pancreas attached to the bottom of the spleen.

4.9. Using flat-tipped forceps, retract the tail of the pancreas laterally.

4.10. Pick up one finely minced tumor piece from the donor mouse with the tip of a fine (6-0) sterile suturing needle and position it using sterile forceps.

4.11. While gently retracting the tail/body of the pancreas laterally, insert the needle of the suture containing tumor fragment into the tail of the pancreas. Pass the suture slowly through the tissue, holding the tumor in contact with the pancreatic tail. Apply one or two additional sutures depending on the fragment's orientation in relation to the tissue.

4.12. Remove the needle completely from the tissue and keep the pancreas/spleen externalized for an additional 60 s while inspecting for any signs of hemorrhage or leak. Then, internalize them gently into the abdominal cavity using blunt forceps.

4.13. Close the abdominal musculature using a 6-0 absorbable suture with either a continuous or interrupted stitch, and close the overlying skin using a 3-0 to 6-0 non-absorbable interrupted suture. Discontinue the anesthesia at this point.

4.14. Allow the mouse to recover in its home cage with free access to food and water. Place the cage on a heating pad to facilitate recovery. Monitor vital signs such as breathing, perfusion *etc.,* during the recovery process.

4.15. Confirm the signs of righting reflex once the mouse recovers, and then return the cage can to regular housing.

4.16. Administer buprenorphine 0.05–0.1 mg/kg 8–12 h after the surgery, and every 8–12 h after the procedure as needed for signs of pain.

4.17. Monitor tumor growth using an in vivo bioluminescence imaging system (see the **Table of Materials**).

4.17.1. Follow tumor growth by imaging starting on day 4 after orthotopic implantation, and perform imaging twice a week.

4.17.2. On the day of imaging, administer (intraperitoneal injection) 30 mg/kg D-luciferin to an anesthetized (2–3% isoflurane in oxygen (2 L/min)) recipient mouse 10 min prior to imaging.

4.17.3. Image for luciferase activity using the luminescence setting without any emission filters for a minimum of 5 s exposure in a luminescence imager with auto-fluorescence free heated stage and while still maintaining the anesthesia for the mice.

5. IRE of SQ tumors

5.1. Prepare the materials required: square wave electroporator, safety foot switch, electrodes (needle array versus tweezer-trode), and adaptors. Keep sterile sutures (non-absorbable 4-0), alcohol swabs, hair clippers, depilatory cream, eye lubricant, cotton gauze, buprenorphine, and anesthetics also nearby.

5.2. Sterilize the tweezer or needle array electrodes using gas sterilization. Autoclaving is not recommended. Use a glass bead sterilizer to sterilize the electrodes between animals.

5.3. Administer buprenorphine 0.05–0.1 mg/kg to the tumor-bearing mice 30 min prior to the procedure.

5.4. Follow steps 4.4–4.5 to successfully induce anesthesia in mice once the SQ tumor implant reaches 5 mm diameter.

5.5. Place the anesthetized mouse on its side to access the SQ tumor on the flank. Remove the hair using clippers and clean skin using an alcohol swab.

5.6. For SQ tumors, use 2-needle array electrodes. Elevate the skin directly under the tumor site using forceps and insert the electrodes through the skin parallel to the body making sure they do not penetrate the peritoneal cavity. Once through the skin, position the electrodes in such a way that they bracket the tumor.

5.7. Program the electroporator to deliver 100 µs pulses at a frequency of 1 Hz at 1,500 V/cm for a total of 150 pulses. Deliver the pulses using the foot pedal. Separate each set of 10 pulses by 10 s, in order to allow for heat dissipation and confirm the correct position of the electrodes.

NOTE: Without the use of paralytic agents, mice will experience muscle contractions with IRE that can cause displacement of the electrodes unless manually secured.

5.8. Remove the electrodes after complete dosing, which should not exceed 200 s. Record the actual voltage delivered, which is displayed on the electroporator. Allow the mice to recover following IRE as per steps 4.14–4.16.

6. IRE of orthotopic tumors

NOTE: IRE of orthotopic tumors involves a second survival surgery on the same mouse thus requiring special approval from local IACUC before beginning.

6.1. Prepare required materials: autoclave the surgical tools such as scissors, scalpels, needle drivers, pointed forceps, and flat tipped forceps. Keep the following nearby as well: sterile sutures (absorbable 6-0 and non-absorbable 4-0), alcohol swabs, hair clippers, depilatory cream, eye lubricant, cotton gauze, 10% povidone iodine solution, surgical drapes, heating pads, square wave electroporator, safety foot switch, electrodes and their adaptors, buprenorphine, and anesthetics.

6.2. Assess the mice for orthotopic tumor growth by *in vivo* imaging (see step 4.17) by injecting 30 mg/kg of D-Luciferin intraperitoneally.

NOTE: The orthotopic tumors are ideal for IRE treatment 8 to 10 days post implantation when the tumors are clearly visible and still confined to the pancreas as can be seen on the Luciferase luminescence images (see the **Representative Results**).

6.3. Follow steps 4.4–4.9, to locate the implanted tumor. If the tumor is not easy to locate, use blunt nosed forceps to move the stomach and spleen gently to identify the tumor.

6.4. Externalize the tumor with blunt nosed forceps and capture the tumor tightly with the platinum electrodes of the tweezer-trode. Deliver the electroporation pulses as programmed in step 5.7 using the square wave electroporator in sets of 10 pulses controlled by the foot switch.

6.5. Keep the tumor externalized for at least 60 s post IRE to monitor for any signs of hemorrhage. Insert the tumor back into the abdominal cavity and close the incision as described earlier (steps 4.13–4.16) and monitor the mouse until it recovers.

6.6. Monitor the effects of IRE on tumor growth using *in vivo* luciferase imaging as per step 4.17.

REPRESENTATIVE RESULTS:

We followed the procedure described above and generated SQ tumors on 5–6 week old wild type C57BL/6 mice inoculated with 1 x 10^6 cells with 50% BMM. When the tumor size reached 5–6 mm in diameter, a few of the mice were euthanized, their tumors were excised, and implanted orthotopically in a recipient C57BL/6 mice. IRE was performed 10 days post implantation as shown in the timeline on **Figure 1**. IRE was performed on the remaining mice bearing SQ tumors.

In SQ tumors, the IRE voltage and pulse duration were kept constant at 1,500 V/cm and 100 µs, respectively, but the number of pulses varied. **Figure 2** shows that the SQ tumors in a few mice regressed completely after 150 pulses of IRE but not as well with 75 pulses. In total, 4 out of 9 mice showed complete tumor regression after 150 pulses of IRE. Histological analysis of tumor tissue 1 week post IRE showed large regions of central tumor necrosis that were not seen in control untreated tumors. This necrotic core was flanked by live tumor tissue in cases of incomplete tumor regression (**Figure 3**). Successful implantation of orthotopic tumor was also achieved, and the growth rate was monitored using *in vivo* bioluminescence imaging showing tumor at day 10 and day 15 (**Figure 4**) after implantation. IRE at 150 pulses also proved to be effective in orthotopic tumors (**Figure 5**) showing reduced tumor volumes. Overall, these results demonstrate the ability of this model to simulate effects of IRE in an immunocompetent mouse model, thereby providing a platform to test various IRE conditions and combinations to treat PC.

FIGURE LEGENDS:

Figure 1: Schematic representation shows the time course of tumor implantation and IRE. For SQ tumors, IRE is performed as soon as the tumor reaches 5–6 mm in diameter.

Figure 2: Luciferase-bioluminescence imaging shows a reduction in tumor growth post IRE in SQ model. KPC-Luc cell line constitutively expresses luciferase making it feasible to monitor tumor growth in response to IRE in real-time. Day 14 bioluminescence imaging post IRE shows the complete regression of tumor in one of the mice treated with 150 pulses of IRE, whereas incomplete regression was seen with 75 pulses of IRE when compared to the untreated control.

Figure 3: Hematoxylin and eosin staining of tumor tissue shows the changes in the tissue architecture post IRE. SQ tumors exposed to IRE showed large regions of necrosis centrally, surrounded by regions of live tissue indicating inadequate IRE coverage in some cases.

Figure 4: Bioluminescence imaging shows the successful implantation of orthotopic tumor and its growth over time. Images were taken using a commercial bioluminescence imaging

instrument with the luminescence captured at 1 min exposure. The mice were injected with 30 mg/kg of D-luciferin solution intraperitoneally 10 min before imaging. Mice were kept anaesthetized using 2% isoflurane during the procedure.

Figure 5: IRE induces tumor growth reduction in orthotopic PC tumors. (**A**) Bioluminescecnce images of mice harboring orthotopic PC showed reduced luciferase signal 7 days post-IRE compared to sham surgery suggesting reduced live tumor burden as a result of IRE. (**B**) Mean volume of excised orthotopic tumors (+ standard error) in control mice vs. mice that underwent IRE.

DISCUSSION:

In this study, we have demonstrated an immunocompetent mouse model for PC that can be used to study the effects of IRE on the tumor growth. Currently, IRE is being used as a non-thermal ablation technique only in highly selected locally advanced PC patients who do not have distant disease progression after months of preoperative therapy. Its use has therefore been limited because most patients with locally advanced PC develop distant metastatic disease[20]. This model will serve as a basis for studies to evaluate the effects of IRE on PC using various extended treatment parameters and combinations.

The most important thing to consider during the protocol is the size of the tumor at which IRE is performed. The commercially available probes for mice models are limited by their electrode distance (5–10 mm). Hence, in tumors significantly larger than the electrode distance, incomplete ablation takes place. For SQ tumors, the use of 2-needle array electrodes over tweezer-trodes is recommended as the skin surrounding the tumor increases resistance to the flow of current in tweezer-trodes. The size limitation of the 2-needle array electrodes can be overcome by performing the IRE at various depths and angles in the same tumor; however, this approach makes it difficult to treat tumors uniformly. Alternatively, for larger tumors (up to 10 mm), an incision can be made on the skin below the tumor to a length slightly longer than the tumor diameter. The tumor can then be externalized through the incision by inverting the overlying skin using forceps. The tumor can then be treated with tweezer-trodes, similar to the orthotopic tumors described above. The electroporated tumor can then be reinserted under the skin, and the skin sutured with a 4-0 non-absorbable suture as an interrupted stitch. However, the effects of the incision may confound the effects of IRE, and we have found that performing IRE when the tumors are within the working distance of the two-needle array electrodes (~ 5–6 mm) provides the best standardization.

Regardless of the approach, the number of pulses needs to be determined empirically depending on the tumor type. Published studies have utilized between 150–300 pulses at this voltage[17]. However, we determined the optimal number of pulses in a preliminary dose-response experiment. There are models to predict the zone of complete ablation based on voltage and electrode distance, but tumor types can vary greatly in vascularity and fibrosis, which may affect the response to electroporation[21].

Each set of 10 pulses was separated by 10 s in order to avoid the heating effects that occur if too many pulses are delivered too quickly. Over-treatment can result in thermal ablation, which may prevent accurate characterization of the effects of non-thermal IRE. The 10 s interval also allows us to frequently confirm electrode positioning accurately since muscle contractions can cause electrode displacement. Our IACUC has not yet allowed the use of paralytic agents in small animals, which can greatly reduce muscle contractions during IRE. In case of orthotopic tumors, the tweezer-trodes allows us to hold the tumors tightly and have a larger electrode surface area compared to the needle electrode.

Although IRE is currently only used as an ablative procedure in the clinical setting, electroporation in general has a wide spectrum of applications ranging from nerve and muscle activations to delivery of various drugs and oligonucleotides. By carefully analyzing the pathophysiological changes occurring during and after IRE using this model, it is possible to develop countless therapeutic strategies for PC.

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The authors have no relevant financial disclosures.

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