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TITLE:

Establishment of Liver Cancer Patient-derived Xenograft Models

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KEYWORDS:

patient-derived xenograft; anti-cancer; liver cancer; scid mice; sorafenib; preclinical

SUMMARY:

Here, we present a protocol to establish liver cancer patient-derived xenograft models for the preclinical study of novel anticancer drugs.

ABSTRACT:

Patient-derived xenograft (PDX) models are established by transplanting immune-compromised mice with tumor samples from cancer patients. The application of PDX models has facilitated the development of anticancer drugs in preclinical studies. In this article, we present a method to establish a liver cancer PDX model. Human liver cancer tissues are subcutaneously injected into scid mice to generate a bank of tumors, which are subsequently passaged into different generations of mice to maintain the liver cancer PDX models. The liver cancer PDX models mostly resemble their original tumor properties as determined by immunohistochemistry analysis and western blot assay. Treatment with sorafenib, a Food and Drug Administration (FDA)-approved standard first-line drug that has been used for the treatment of unresectable liver cancers, suppresses the tumor growth in the liver cancer PDX model. Although there are limitations to this liver cancer PDX model, it has helped scientists to investigate, in preclinical studies, novel therapies for liver cancer treatment which are more precise and clinically relevant.

INTRODUCTION:

PDX models are established by transplanting immune-compromised mice with tumor samples from cancer patients[1,2]. PDX models have been established in various types of cancers, including breast cancer, lung cancer, liver cancer, pancreatic cancer, and so on[3]. PDX models retain the

genomic, histologic, and biological properties of the corresponding primary tumors. More importantly, the response of PDX models after anticancer drug treatment has been found to be associated with the clinical outcome of cancer patients, which is important for healthcare professionals when making therapeutic decisions for the better management of cancer. Ruiz et al.[4] developed a triple-negative breast cancer 1, early onset (BRCA1)-mutated PDX model and depicted a link between transcription factor 4 expression and breast cancer chemoresistance. Yao et al.[5] showed that epidermal growth factor receptors (EGFRs) and RAF inhibition demonstrate synergistic antitumor activity for colorectal cancer PDX models with a KRAS or BRAF mutation. Nicolle et al.[6] showed that PDX models from pediatric liver cancer predict tumor recurrence and advise clinical management. In this regard, PDX models have been regarded as the most suitable preclinical models for anticancer development and the research into the mechanisms of cancer development.

Liver cancer is the third leading cause of cancer-related deaths worldwide. According to the statistics, an estimated 30,000 new cases and 40,000 deaths occurred in the United States in 2017, and China has the highest incidence rates for liver cancer[7]. As far as we know, sorafenib is the only FDA-approved standard first-line drug that was used for the treatment of unresectable liver cancers. Unfortunately, due to the multiple mutations in the liver cancer, sorafenib only improved the overall survival of liver cancer patients by around 3 months[8]. Drugs that targeted the specific mutation would provide more effective treatments for liver cancer patients. Therefore, the preclinical validation of the effectiveness of drugs by using PDX models will facilitate the anticancer treatment for liver cancer.

The first liver cancer PDX model was reported in 1996[9]. However, due to the low engraftment rate, the progression of liver cancer PDX models developed very slowly. Recently, due to the wide application of PDX models and improvement of experimental protocols, the engraftment rate of PDX models was increased to around 40% and many liver cancer PDX models have been used for the screening of anticancer drugs for liver cancer[10]. Although liver cancer PDX models have been extensively applied in the research, there are still challenges, such as the long time (2–4 months) it takes liver tumors to engraft and the high rate of engraftment failure. In this regard, it is important to refine and improve the experimental protocols for liver cancer PDX models to increase the engraftment rates.

In our laboratory, we have previously developed an experimental protocol to generate liver cancer PDX models with a good engraftment rate, and the establishment of the PDX models made it possible to reveal some important anticancer mechanisms[11]. In this article, we describe in detail a method for the generation of a liver cancer PDX model with a high engraftment rate.

PROTOCOL:

This protocol has been conducted at the University of Hong Kong with approval from the Institutional Review Board of the University of Hong Kong/Hospital Authority of Hong Kong (UW05-3597/I022).

1. Preparation of the patients' tumor sample (~2 cm x 2 cm)

1.1. Prepare Hank's balanced salt solution (HBSS; 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.4 mM $MgSO_4$-$7H_2O$, 0.5 mM $MgCl_2$-$6H_2O$, 0.3 mM Na_2HPO_4, 0.3 mM KH_2PO_4, 6 mM glucose, 4 mM $NaHCO_3$) containing 10% penicillin and 10% streptomycin and collect 20–25 mL into a sterile collection tube for tumor specimen collection.

1.2. Retrieve tumor samples in a sterile tube containing Dulbecco's modified Eagle's medium (DMEM)/F12 on ice.

NOTE: The liver cancer tumor tissues are removed by a surgeon and collected by a pathologist. The tissues are collected into a sterile collection tube containing DMEM/F12 medium.

1.3. Bring the tumor samples to the animal facility for implanting them into immunocompromised mice.

2. Tissue processing

NOTE: Perform this step in a laminar flow hood to maintain sterility.

2.1. Place the tumor sample into a sterile Petri dish (100 mm in diameter x 13 mm in depth) from the collecting tube and rinse the tumor tissues 2x with cold HBSS solution.

2.2. Use autoclaved small scissors and forceps to cut the tumor tissues into 1 mm x 1 mm pieces. Transfer the tissue pieces to an autoclaved 1.5 mL microcentrifuge tube filled with 300 µL of gelatin solution. Keep the tube on ice.

2.3. Alternatively, for the preparation of tumor cells from tumor tissues, rinse the collected tumor samples 2x with 0.5 mL of ice-cold HBSS and incubate in DMEM/F12 medium containing 10% penicillin and 10% streptomycin for 30 min at 37 °C.

2.4. Slice the tumor tissues into 1 mm x 1 mm pieces by using autoclaved small scissors and forceps. Digest the tumor tissues with 0.02 mg/mL collagenase IV for 45 min at 37 °C.

2.5. Filter the tissues through 100 µm and 40 µm meshes. Lyse the red blood cells by incubating them with ammonium chloride for 30 min at 37 °C. Collect the tumor cells by centrifugation at 800 x *g* for 10 min; then, rinse them with DMEM/F12 1640 medium.

2.6. Culture the isolated cells (1 x 10^6) in 10 mL of DMEM/F12 medium containing 10% fetal bovine serum for 24 h.

NOTE: Use these tumor cells for the subcutaneous injection into the mice (see section 3).

3. Implantation of patient-derived tumor xenografts

3.1. Use eight 6- to 8-week-old male scid or NOD/scid mice for each separate patient-derived tumor tissue.

NOTE: The mice injected with the original human tumor are designated as the F0 generation, and then the next generation is generated by injection tumors from the previous generation and is designated as F1, F2, F3, and so on.

3.2. Load one piece of tumor from the gelatin or load cells from the culture medium into autoclaved 12 G trocars and make sure that the tumor is completely pushed into the trocar.

3.3. Perform anesthesia in an anesthesia box placed in a laminar flow hood. Place eight mice into the anesthesia box, which is connected to an isoflurane anesthesia machine (2%–3% isoflurane with 3%–4% oxygen for the maintenance of anesthesia).

3.4. Pinch a mouse's toe for the observation of pedal reflex to ensure that the mouse is fully anesthetized. Once a lack of pedal reflex is observed, place the mouse on a sterile place and inject the tumor or cells into the middle dorsal neck region. Do this with autoclaved 12 G trocars by sliding the trocar down subcutaneously until the flank region is reached.

3.5. Before the mouse is fully awake, inject buprenorphine (0.1 mg/kg) subcutaneously. Next, place each mouse individually into the cage and monitor it until it is awake and moving.

4. Establishment of patient-derived tumor xenograft bank

4.1. Monitor the growth and health of the mice weekly. Track tumor sizes, the data of tumor injection, and the health of the mice. Using a caliper to measure the dimension of the tumor, calculate the tumor volume based on the following formula: (width × width × length)/2.

4.2. When the tumors are around 500 mm^3 (which takes about 3 weeks), anesthetize the mice (10–12 mice) with isoflurane (2%–3%) followed by cervical dislocation.

4.3. Use the above-mentioned procedures (sections 2 and 3) to collect 10–12 tumors for passage into the next generation and collect the leftover tumor for future use.

4.4. Maintain the remaining mice (8–10 mice) until a new generation of mice has tumors of around 500 mm^3.

5. Determination of the effects of sorafenib on the tumor growth of patient-derived tumor xenografts

5.1. When most F1 tumors are larger than 20 mm^3, start the treatments with a vehicle (saline, 20 mL/kg) or sorafenib (30 mg/kg) and monitor the tumor volumes and body weights of the mice approximately 3x per week.

5.1.1. Randomly and blindly divide the F1 tumor-bearing mice into a vehicle group (20 mL/kg) and a sorafenib (30 mg/kg) group, making sure each group consists of six mice, each with at least one PDX tumor.

5.1.2. Administer the vehicle (saline, 20 mL/kg) or sorafenib (30 mg/kg) to the mice via oral gavage, 2x a day.

5.1.3. Treat the mice for 2 weeks and euthanize them after another 2 weeks with an overdose of pentobarbital sodium (50 mg/kg).

5.2. Make a small incision (1–2 cm in length) with autoclaved small scissors and forceps to gently and completely remove the PDX tumor from the mammary fat pad.

5.3. Use the collected tumor tissues to perform western blot for the detection of cyclin-dependent kinase 1 (CDK1), phosphoinositide-dependent kinase 1 (PDK1), and beta-catenin proteins and immunohistochemistry for biomarkers analysis (Hep-Par1, CK7, CK20, and CEA)[11].

REPRESENTATIVE RESULTS:

An overview of the liver PDX model protocol is shown in **Figure 1**. A patient-derived tumor was obtained after surgery and immediately injected into the mice subcutaneously. After the injection, the tumors were left to grow in the mice. The tumors were also observed in subsequent generations and eventually expanded for treatment studies.

Figure 2A represents the mouse model of F1 PDX tumors. The immunohistochemistry analysis of biomarkers (Hep-Par1, CK7, CK20, and CEA)[11] showed that the expression pattern of these biomarkers in PDX tumor models mostly resembled that of their original tumor tissues (**Figure 2B**). In addition, the western blot analysis showed that the levels of CDK1, PDK1, and beta-catenin are specifically similar between the clinical tissues and paired PDX models (**Figure 2C**).

After the PDX mice were treated with saline (20 mL/kg) or sorafenib (30 mg/kg) for 2 weeks, the mice were killed after another 2 weeks and the tumor tissues were dissected. **Figure 3A** shows the images of the dissected tumor tissues from PDX mice by using tumor tissue from one patient. The growth curves of the tumor in PDX models are shown in **Figure 3B**, and the results show that the tumor growth was suppressed by more than 50% after sorafenib treatment when compared to the control group (**Figure 3B**). In addition, there was no significant difference in body weight between the control and the treatment group (**Figure 3C**).

FIGURE AND LEGENDS:

Figure 1: Establishment of liver PDX tumor model. The diagram of the established PDX tumor model (F0 and F1 generation) and the targeted drug administration schedule. This figure has been modified from Wu et al.[11].

Figure 2: F1 PDX tumor grafts resemble the patient tumors from which they are derived. (**A**) The images represent the mouse model of F1 PDX tumors. (**B**) Hep Par1, cytokeratin 7 (CK7), cytokeratin 20 (CK20), and CEA staining for F1 PDX tumors and paired clinical tissues. The scale bar = 100 µm. (**C**) In comparison to the protein expression levels (CDK1, PDK1, and beta-catenin), PDX tumors highly mimic the paired clinical tumors analyzed by western blot. This figure has been modified from Wu et al.[11].

Figure 3: The effectiveness of sorafenib on PDX tumors. (**A**) The growth curves of PDX models after different treatments. (**B**) The representative images of the dissected tumor tissues from the PDX models. (**C**) The body weight of the PDX models after different treatments. This figure has been modified from Wu et al.[11].

DISCUSSION:

Liver cancer has very a low survival rates and a high probability of metastasis, making it one of the most aggressive cancers. In this article, we described a detailed protocol for the generation of an improved liver cancer PDX model. In this model, patient-derived tumors were injected into scid mice, passaged, and subsequently applied in the evaluation of the anticancer effects of sorafenib. More importantly, this liver cancer F1 PDX model mostly retains the characteristics of the original clinical tumor, has similar drug responsiveness, and is biologically stable. For the successful establishment of the liver cancer PDX model, several key factors should be considered. First, a professional clinical team was required to identify the pathology of the tumor tissues and a clinical record of the patients and to ensure the quality of the collected liver cancer tissues. Second, the fresh tumor tissues should immediately be kept in the proper medium with antibiotics on ice to avoid tumor necrosis. Third, a research team with good technical skills for processing the tumors and injecting the tumor tissues into nude mice is the most important. These factors are important for the successful establishment of liver cancer PDX models and the subsequent anticancer drug evaluation.

In our laboratory, the success rate of establishing a liver cancer PDX model was around 100%. Several factors have indeed affected the success of the establishment of liver cancer PDX models. For example, the collected tumor tissues were undergoing necrosis. Sometimes, the collected tissues were very small, and the tissues were not enough for multiple mice injection, which decreased the chance of success. In addition, some tissues collected from the clinical site were just normal tissues without tumor tissues after histology evaluation. In this regard, it is essential to have a reliable surgical team and experienced pathologist on site to ensure the quality of the collected tumor tissues. In addition, the collected tissues should be annotated with detailed clinical characteristics, which is important for the evaluation of anticancer effectiveness and improving the therapeutic approach for the specific patient.

The liver cancer PDX model has been used in the determination of drug efficacy, drug resistance mechanisms, and the effectiveness of drugs on cancer stem cell populations in various studies. Yang et al. revealed that activating Janus kinase 1 mutation may predict the sensitivity of the Janus kinase-signal transducer and activator of transcription inhibition in hepatocellular carcinoma (HCC) by using the liver cancer PDX model[12]. Nicolle et al. developed the pediatric liver

cancer PDX preclinical platform, which could accelerate the identification and diversification of anticancer treatment for aggressive subtypes of pediatric liver cancer[6]. Du et al. showed that AMG337, a highly selective small molecule Met inhibitor, inhibited tumor growth in the Met-high-expressing liver cancer PDX model[13]. By using the liver cancer PDX model, we found that the Notch inhibitor PF-03084014 inhibited HCC growth and metastasis via the suppression of cancer stemness due to the reduced activation of notch1-stat3[14]. In the subsequent study, we demonstrated that blocking CDK1/PDK1/beta-catenin signaling by CDK1 inhibitor RO3306 increased the efficacy of sorafenib treatment by targeting cancer stem cells in the liver cancer PDX model[11]. All in all, these data implicate the importance of applying liver cancer PDX models in the development of anticancer drugs, which may significantly improve the clinical outcomes of patients with liver cancer.

As far as we know, there are several limitations to the PDX model. In the liver cancer PDX model, the human stroma from the freshly collected tumor tissues was replaced with the mouse stroma after implantation, which is a problem when mouse ligands failed to activate the receptors on the human tumor cells[15]. Another problem for this model is that it does not make it possible for scientists to study the role of the immune system in cancer development[16], which prevents the investigation of immune-targeted agents in the PDX model.

In conclusion, we described a method of establishing a liver cancer PDX model that is useful in determining the therapeutic efficacy of anticancer drugs. Although there are limitations to this PDX model, the liver cancer PDX model makes a most precise and clinically relevant investigation of novel therapies in preclinical studies possible.

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DISCLOSURES:

The authors have nothing to disclose.

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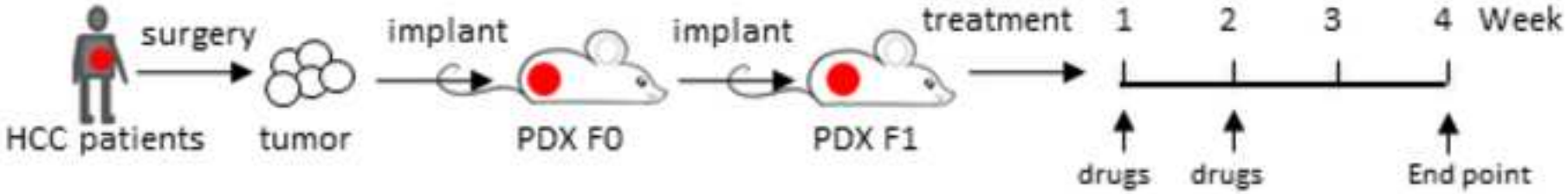
Fig.1



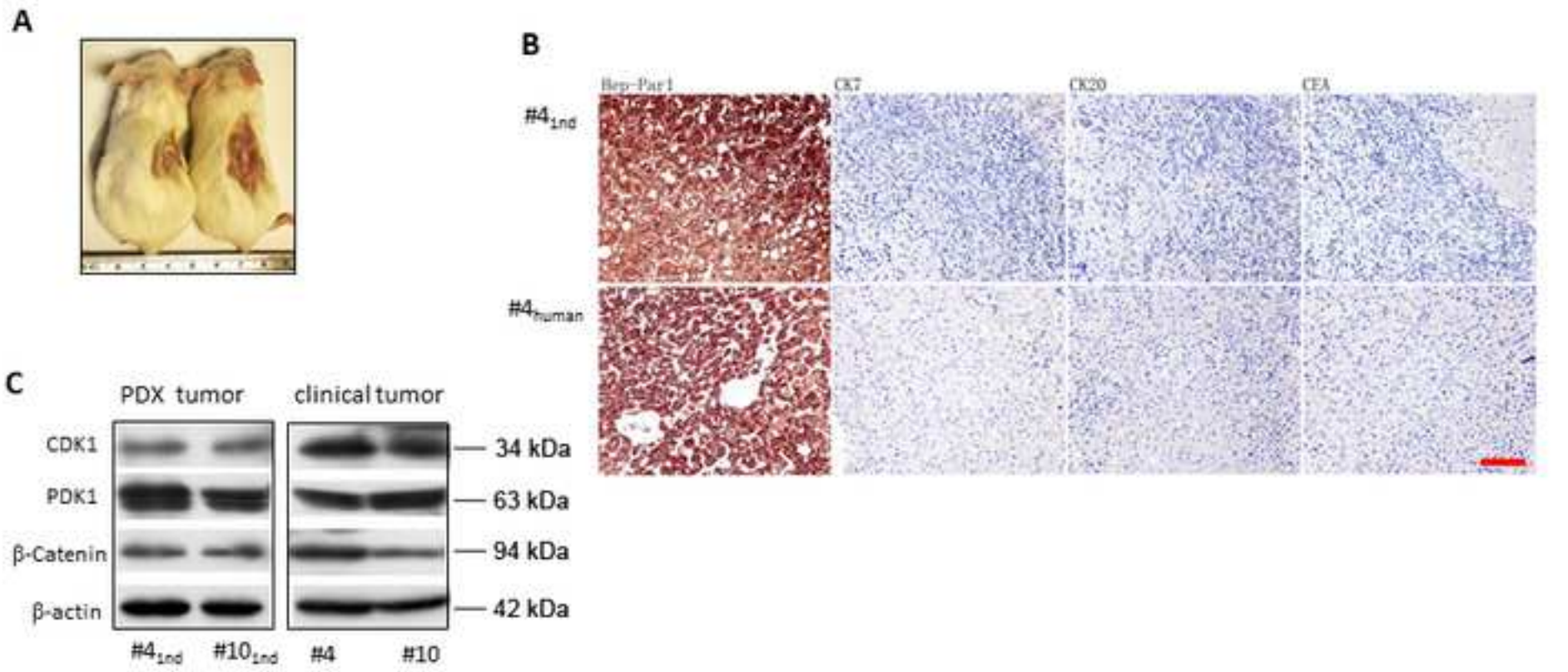


Figure.3 R1

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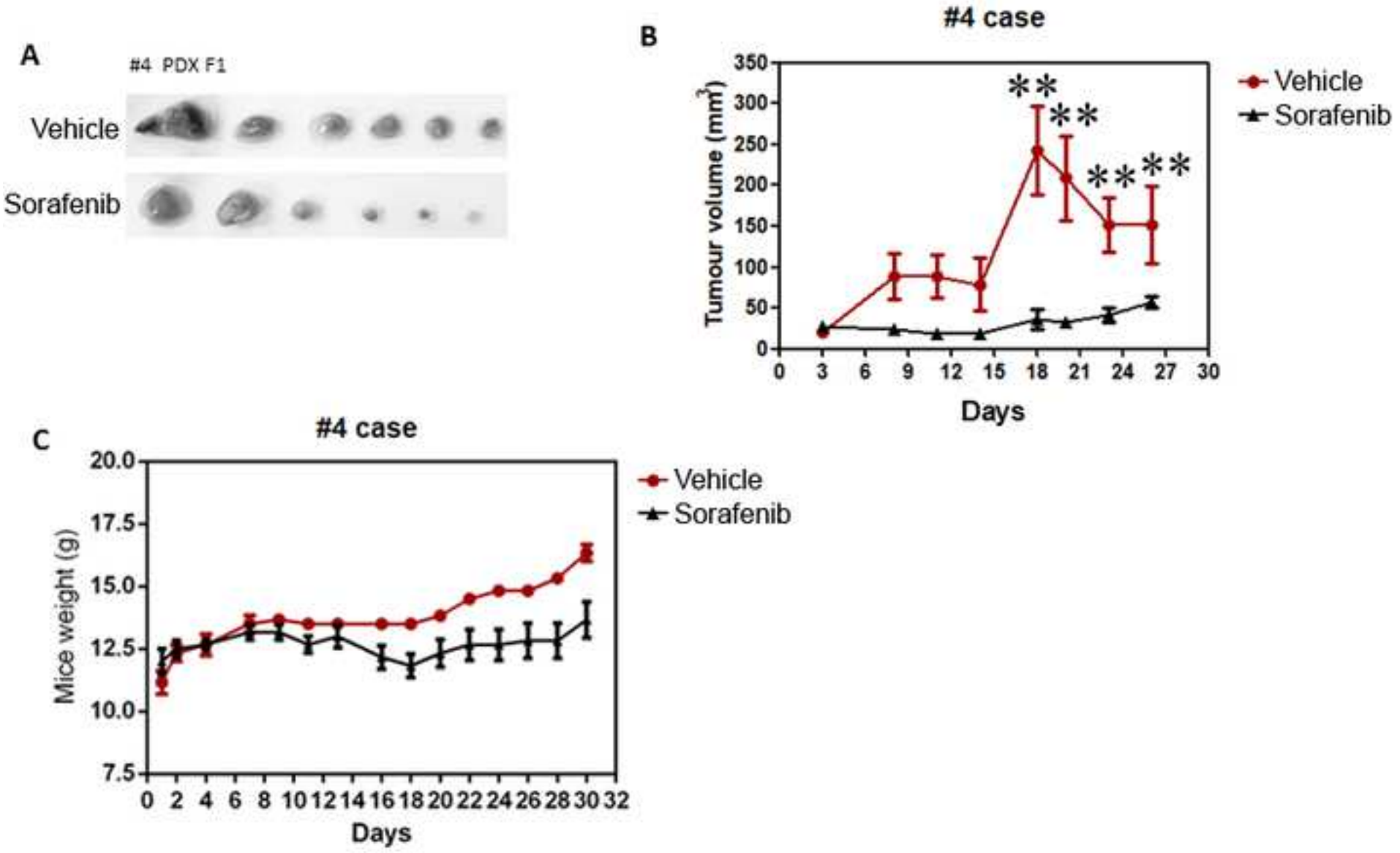

Table of Materials

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Name	Company	Catalog Number	Comments
1.5 ml microcentrifuge tube	Pipette	22363204	
Ammonium chloride	Sigma-Aldrich	254134	
Anesthesia bix	Patterson Veterinary		
Anesthesia machine	Patterson Veterinary		
Buprenorphine	Sigma-Aldrich	B-044	
Calipers	Flowler	54-100-167	
Collagenase IV	Thermo Fisher Scientific	17104019	
DMEM/F12	Thermo Fisher Scientific	11320033	
Fetal bovine serum	Sigma-Aldrich	F2442	
Forceps	Roboz	RS-5135	
Gelatin solution	Sigma-Aldrich	G1393	
Hank's Balanced Salt Solution	Thermo Fisher Scientific	14025076	
Isoflurane	Vet one	1038005	
Penicilin	Sigma-Aldrich	13752	
Scissors	Roboz	RS-5881	
Streptomycin	Sigma-Aldrich	S6501	
Trocars	Innovative Research of America	MP-182	
Weight scale	Ohuas	Scout Pro SP601	

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