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Establishment of an orthotopic nude mouse model for recurrent pancreatic cancer after complete resection: an experimental animal study

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Purpose: This study created a nude mouse model to study pancreatic cancer recurrence. Circumstances leading to the highest recurrence rates after curative surgery were also analyzed.
Methods: A total of 135 nude mice were divided into 3 groups: sham, metastasis, and resection (45 mice in each group). In sham and resection groups, AsPc-1 cells suspended in a synthetic extracellular matrix were injected into the tail of the pancreas of each mouse. In the metastasis group, cells were injected into the spleen. After 3 weeks, the resection group underwent distal pancreatectomy and the metastasis group underwent diagnostic laparotomy to confirm metastasis. To assess disease recurrence, the resection group was monitored weekly using luminescence imaging. Diagnostic exploration was conducted 3 weeks after surgery. Recurrence rate was evaluated and histological examination was performed for the resection group.
Results: Among 45 mice, 43 developed cancerous masses in the tail of the pancreas without invading adjacent organs 3 weeks after the initial orthotopic injection. Of those 43 mice, one died due to intraoperative bleeding during complete surgical resection. Pancreatic cancer recurrence was observed in 37 of 42 mice (88.1%) at an average of 21.8 ± 2.2 days. Histological examination showed high nuclear pleomorphism and neoangiogenesis.
Conclusion: We developed an efficient model that could demonstrate recurrence after complete resection of pancreatic cancer. By confirming that recurrence occurs after surgery using this protocol, our model is expected to contribute to the development of various treatment strategies.
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Key Words: Mice, Pancreatic neoplasms, Pancreatectomy, Prognosis, Recurrence

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a devastating form of cancer known for its early metastasis, low resectability, high recurrence rates, and resistance to chemoradiotherapy. Despite decades of research, the prognosis for pancreatic cancer remains poor, with 5-year survival rates of less than 5%–10% and a median survival rate of only about 6 months following diagnosis [1]. Surgical resection such as pancreaticoduodenectomy or distal pancreatectomy offers the only hope for a cure. However, most patients ultimately succumb to recurrent pancreatic cancer, with a survival time of less than 21 months [2,3]. Unfortunately, there are currently few effective means of preventing pancreatic cancer

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recurrence, which is responsible for most postoperative deaths. Therefore, there is an urgent need for a new strategy to prevent proliferation of remaining cancer cells, which cannot be confirmed through conventional pathological evaluation after surgical resection. There is also an urgent need to resolve micrometastasis at the time of surgical resection.

Experimental mouse models have been instrumental in advancing our understanding of the pathobiology of PDAC and in preclinical evaluations of potential therapeutic approaches. Several mouse models that target key pathological hallmarks associated with PDAC progression, metastasis, and stromal heterogeneity have been developed [4-7]. Over time, these models have evolved from simple cell line-based heterotopic and orthotopic xenografts in immunocompromised mice to more complex models [8-10].

For animal models to be clinically relevant, they must closely mimic phenotypic features of human diseases. They also need to reflect underlying genetic alterations observed in human counterparts. While murine models of pancreatic cancer have made significant progress in meeting these requirements, certain limitations remain [6]. Traditional animal models used for studying pancreatic cancer have failed to reproduce the common clinical scenario of a surgically resected primary tumor followed by subsequent recurrence of the disease [11].

Our study aims to address this issue by developing a nude mouse model that can efficiently demonstrate the recurrence of pancreatic cancer. We will describe optimal cell lines and their quantities that can produce the most efficient results and identify circumstances where recurrence rates are likely to be highest after complete surgical resection of tumor lesions.

METHODS

Ethics statement

Animal studies were carried out in compliance with the guidelines of the Institute for Laboratory Animal Research of Korea (IRB No: CUMC-2023-0043-01). All experimental procedures were conducted in accordance with the National Laboratory Animal Care guidelines.

Cell line, culture, and laboratory animals

Six- to seven-week-old male BALB/c nude mice (mean body weight, 21.6 g) were obtained from Orient Bio, Inc. The animals were maintained in microisolator cages with sterile bedding and provisions under standardized conditions (12-hour light/dark cycle). AsPC-1 cell line, a human pancreatic adenocarcinoma cell line, was acquired from the Korean Cell Line Bank and cultured in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific) and 1% penicillin-streptomycin antibiotics (Thermo Fisher Scientific). These cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in an incubator. Male BALB/c nude mice utilized in this study were 6 weeks old. They were procured from Orient Bio, Inc., Korea.

Orthotopic injection technique

A total of 135 mice were divided into 3 groups (45 mice for each group): a sham group, a resection group, and a metastasis group. The sham group was intended to observe the natural course of pancreatic cancer. The resection group was designed to study recurrence after surgical resection and the metastasis group was conducted to observe characteristics of pancreatic cancer metastasis (Fig. 1). To anesthetize all groups of mice, they were placed in an isoflurane chamber. Anesthesia was continued on the operating table. Using sterile surgical instruments, a 1-cm incision was made in the skin approximately 1.5 cm left lateral from the midline. This was followed by a 1-cm incision in the underlying abdominal muscle. Forceps were used to locate the spleen, which was gently pulled out of the abdominal cavity. In sham and resection groups, the

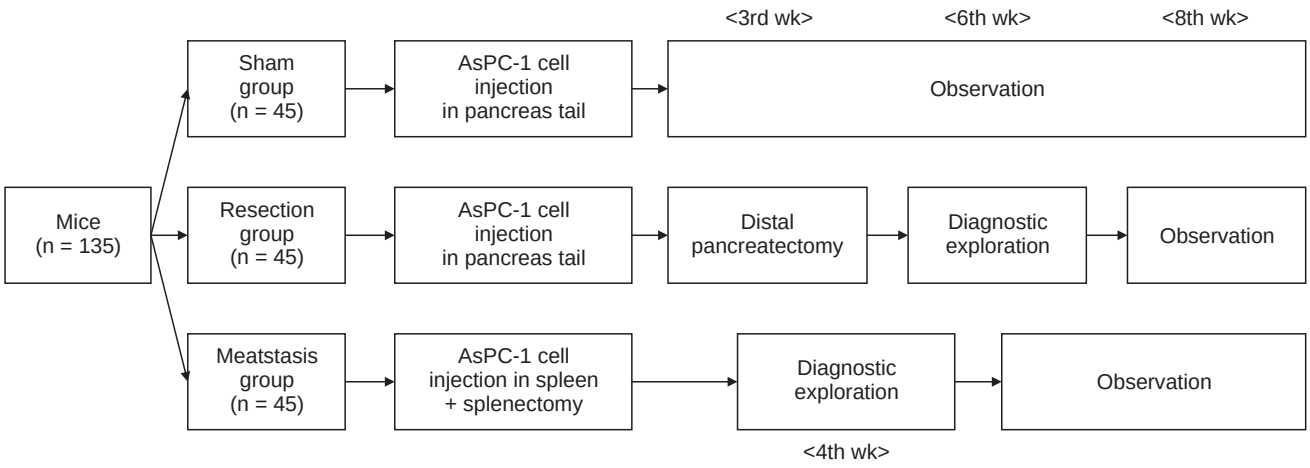

Fig. 1. Study design.

tail of the pancreas adjacent to the spleen was located and 25 μL of AsPC-1 cell suspension (1×10^6 pancreatic cancer cells) was injected into the pancreas using a 29-gauge, 0.3-mL insulin syringe. Care was taken to avoid puncturing the thin dorsal side of the pancreas, which could lead to leakage. After injection, the syringe was held in the pancreas for 30–60 seconds to minimize the chance of cell leakage. The site of injection was inspected to ensure that no leakage occurred. The spleen and pancreas were then returned to the abdominal cavity (Fig. 2). In the metastatic group, 6 μL of the AsPC-1 cell suspension (2.5×10^5 pancreatic cancer cells) was injected into the upper pole of the spleen, followed by splenectomy 15 minutes after spleen injection to fully metastasize pancreatic cells. The abdominal musculature and the external skin of the mouse were closed using an absorbable bard 4-0 suture with a continuous stitch. Finally, the mouse was isolated after inhaling an anesthetic (Fig. 3).

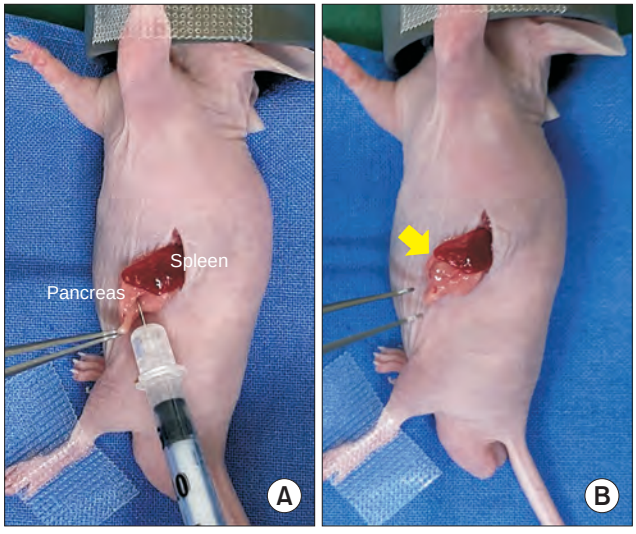

Fig. 2. AsPc-1 cell injection in sham and resection groups. (A) Injecting 25 μL of AsPC-1 cell suspension containing 1×10^6 pancreatic cancer cells into the tail of the pancreas using a 29-gauge, 0.3-mL insulin syringe. (B) Formation of a visible bubble within the pancreatic parenchyma without cell leakage after AsPc-1 cell injection (arrow).

Surgical procedures

Three weeks after the injection, each mouse in the resection group was visually observed to have a mass formed in the pancreas tail under its skin. These mice were then anesthetized, and a longitudinal laparotomy was performed near the spleen to mobilize the tumor with an assistant. Later, during intraperitoneal exploration, it was confirmed that there was no intraabdominal invasion or metastasis to organs other than the pancreas tail mass. The operator utilized surgical clips or 4-0 black silk to ligate the mid-body of the pancreas, ensuring a grossly R0 resection of the tumor. Pancreas transection and splenectomy were then performed using a temperature cautery pen to create a clinically curative surgical resection situation. If pancreatic cancer had progressed throughout the entire pancreas or if peritoneal or liver metastases were found, pancreatic resection was not performed and mice were recorded (Fig. 4).

Bioluminescent tracking of pancreatic cancer recurrence

In a similar manner, a group of mice were injected with AsPC-1 Luciferase/GFP cells orthotopically. Three weeks after injection, these mice underwent distal pancreatectomy. To detect any recurrence of pancreatic cancer, an *ex-vivo* imaging system was used. For this purpose, D Luciferin (LUCK-1G, Gold Biotechnology, Inc.) was prepared at a dose of 15 mg/mL and injected intraperitoneally at 10 μL/g of mice to measure bioluminescence using an IVIS Spectrum Imaging System

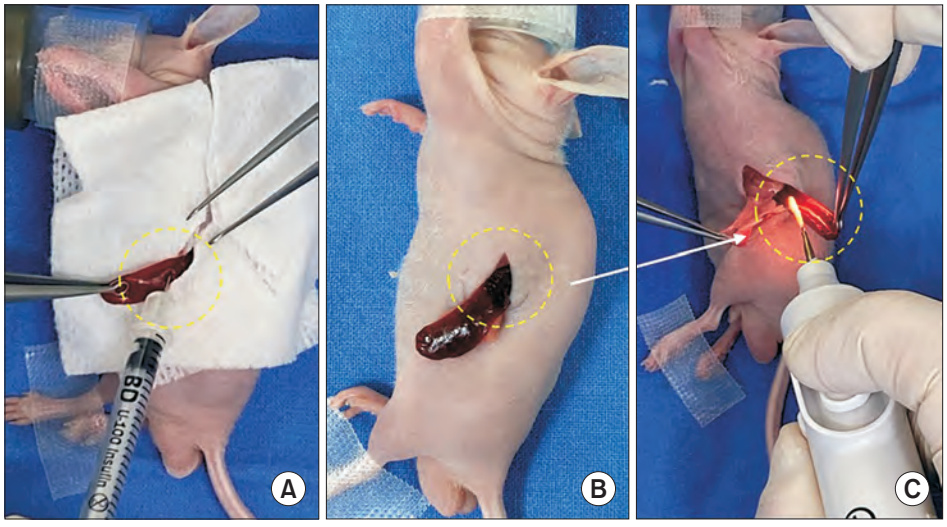

Fig. 3. AsPc-1 cell injection in the metastasis group. (A) Injecting 6 μL of AsPC-1 cell suspension containing 2.5×10^5 pancreatic cancer cells into the upper pole of the spleen (circle). (B) Changes in the spleen parenchyma after cell injection (circle). (C) Performing splenectomy using a cautery pen (circle).

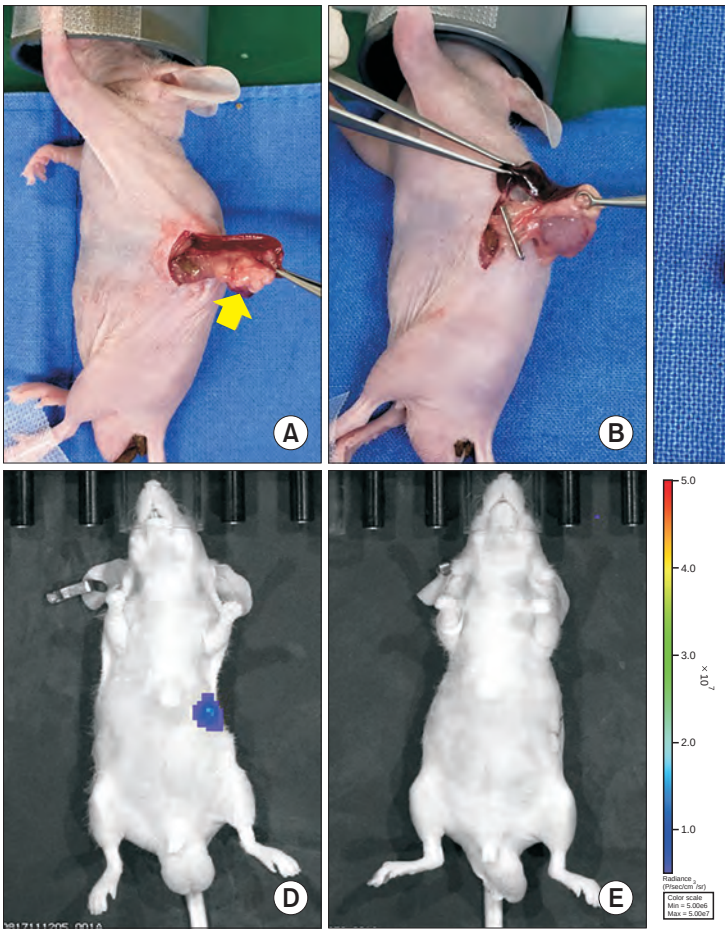

Fig. 4. Surgical procedure for the resection group. (A) Cancer mass formation at the tail of the pancreas without peritoneal or liver metastases (arrow). (B) Ligation of the mid-body of the pancreas using a surgical clip. (C) Specimen after pancreas transection and splenectomy luminescence image of mice. (D) Before curative resection. (E) After curative resection.

(PerkinElmer). Living Image software (PerkinElmer) was used for image quantitation and analysis. Luminescence in mice was measured 3 weeks after tumor resection.

Observation period and sacrifice criteria

Mice were subjected to daily monitoring of their clinical condition and weekly weighing until 6–8 weeks after orthotopic cell injection. At the endpoint, mice were euthanized using a 30%–70% per minute of compressed CO_2 to displace chamber air. Guidelines for euthanasia of rodents were based on humane criteria provided by the Institutional Animal Care and Use Committee which required animals to be euthanized earlier if any of the following occurred: (i) inability to reach food or water for more than 24 hours; (ii) a 20% decrease in normal body weight; (iii) a body condition score typically less than a 2 on a 5-point scale.

Statistical analysis

Continuous data were analyzed using the Student t-test and categorical variables were compared using the Fisher exact test or the chi-square test. Descriptive statistics were conducted using IBM SPSS Statistics ver. 23.0 (IBM Corp.). Data are given as mean ± standard deviation.

RESULTS

Tumor development and progression

We monitored the formation of tumor masses in mice that had been injected orthotopically with AsPC-1 cells in the pancreatic tail or spleen using macroscopic observation and luminescence imaging. Three weeks after injection, tumor developed in the pancreatic tail of each mouse in the sham group. Similarly, in the resection group, tumor was also observed in the pancreas tail of each mouse (Fig. 5). Among the 45 mice, 43 (95.6%) showed tumor growth in the pancreas tail, while the remaining 2 (4.4%) displayed pancreatic tail tumors with invasion of the abdominal wall. In the metastasis group, liver metastases were detected in 40 of 45 mice (88.9%) 3 weeks after spleen injection, with an additional 5 mice (11.1%) showing liver metastases 5 weeks after spleen injection.

Perioperative outcome and tumor recurrence rate in the resection group

Grossly curative resection was successfully performed for 43 of 45 mice (95.6%) in the resection group. However, one of them died due to intraoperative bleeding while dissecting the adhesion around the tumor during surgery. The median

follow-up period in the resection group after surgery was 56 ± 11.7 days. Recurrence occurred in 33 of 42 mice (78.6%) that underwent curative resection at 3 weeks postoperatively. At 4 weeks postoperatively, recurrence was additionally confirmed in 4 animals. Thus, recurrence occurred in a total of 37 of 42 animals (88.1%). The average time to a recurrence rate of 80% or higher was 21.8 ± 2.2 days after surgery. The remaining 5 mice (11.9%) were continuously monitored during the follow-up period. However, no recurrence was observed. Of the 37 recurrences, 25 (67.6%) showed peritoneal masses, 6 (16.2%) had liver metastasis, and 6 (16.2%) recurred in the pancreas stump (Fig. 6).

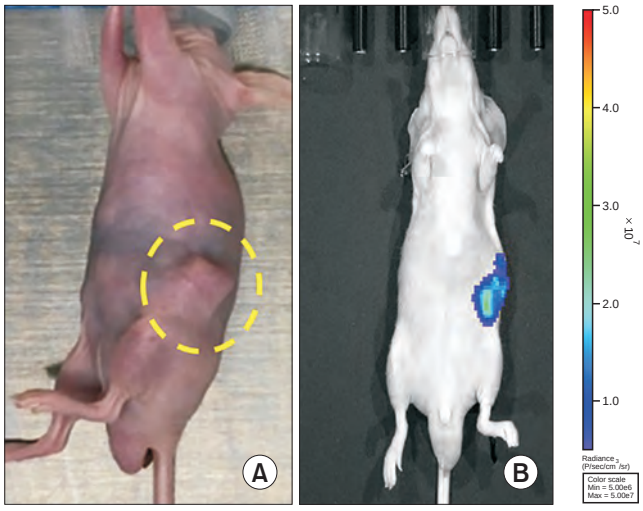

Fig. 5. Mouse tumor mass formation observed on (A) the skin's surface (circle) and (B) in luminescence images of mice at 3 weeks after orthotopically injecting AsPC-1 cells into the pancreatic tail.

Anatomical and histological examinations

Following orthotopic injection, tumors in the pancreatic tail exhibited an irregular shape without adhesions to surrounding organs except in the case where the mouse died from intraoperative hemorrhage. Mice injected with AsPC-1 cells into the spleen showed metastasis to the liver and peritoneum 4 weeks after injection, eventually resulting in the development of massive bloody ascites. We performed a histological examination for all tumors isolated from mice and confirmed that there were no cancer cells left in the resection margin pathologically in all samples. The examination revealed a variety of cell morphologies, with the majority of cells exhibiting either a polygonal or a spindle shape. A fibrous

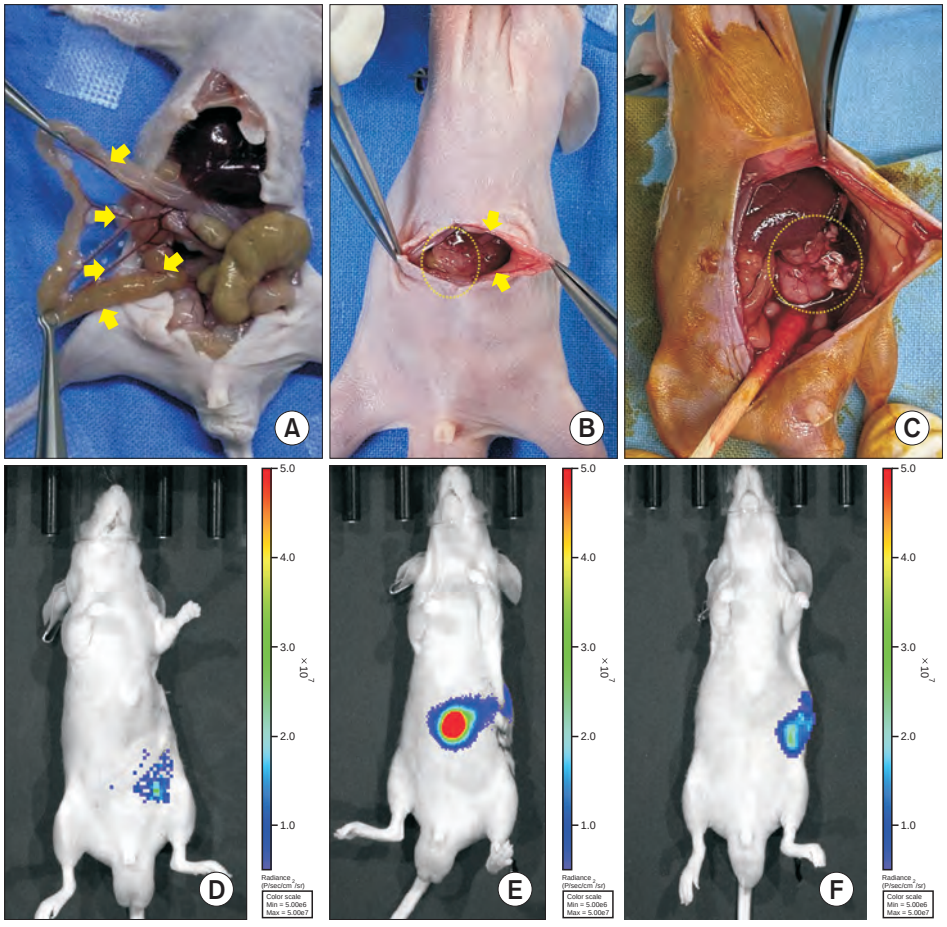

Fig. 6. Recurrence of pancreatic cancer in the resection group after curative resection was observed in the following locations: (A) peritoneal space (arrows), (B) liver (circle and arrows), and (C) the pancreas stump (circle). Additionally, luminescence images showed pancreatic cancer relapse in (D) the peritoneal space, (E) the liver, and (F) the pancreas stump.

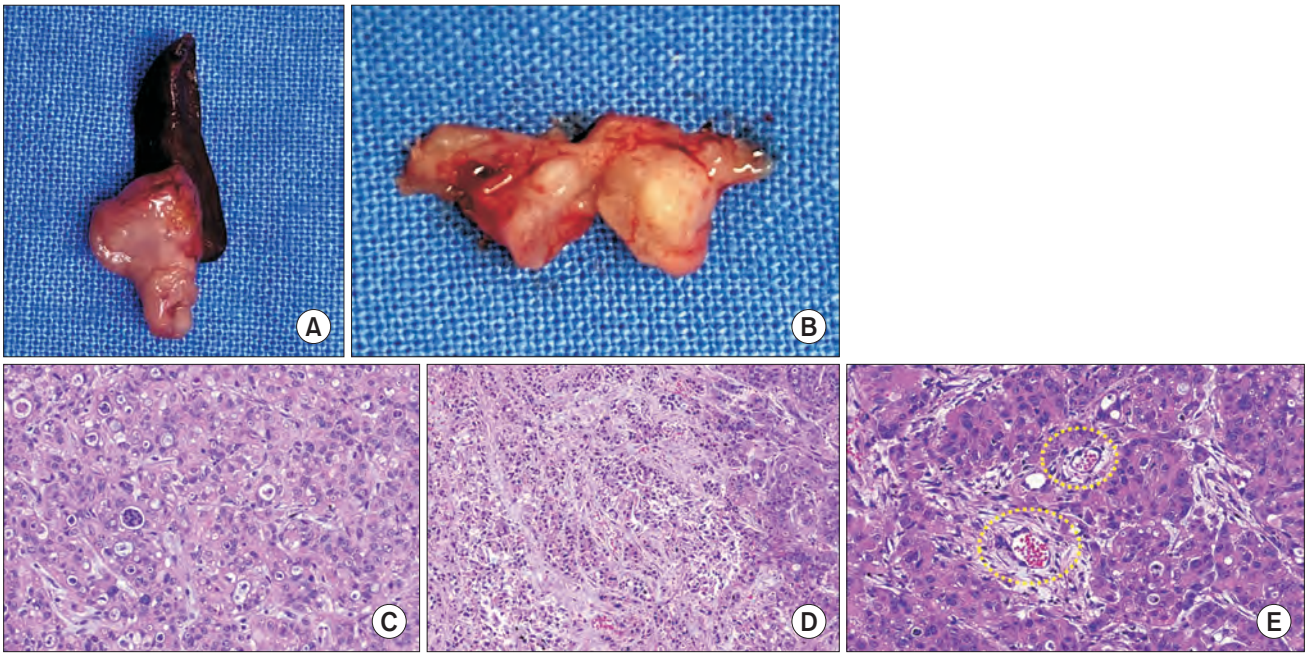

Fig. 7. Pathological examination of the tumor after curative resection revealed the following features: (A) irregular macroscopic appearance with (B) central necrosis; (C) various cell morphologies, with the majority exhibiting either a polygonal or a spindle shape; and (D) presence of fibrous stroma and (E) neoangiogenesis (circles). Panels C–E are H&E-stained sections viewed at ×400 magnification.

stroma was observed surrounding the tumor without showing glandular differentiated cells. Neoangiogenesis was found in 85.6% of all resected specimens. Pathological mitotic figures were also observed in tumors, consistent with a diagnosis of poorly differentiated adenocarcinoma (Fig. 7).

DISCUSSION

At present, complete surgical resection remains the sole long-term treatment option for PDAC. However, 60%–80% of patients who undergo curative resection experience tumor recurrence [12,13]. One potential explanation for this phenomenon is that the persistence of residual tumor within the resection margin as indicated by 'field effect' mutations can affect normal cells in the residual pancreatic tissue [14]. In addition, even if microscopic curative resection of pancreatic carcinoma was completed, micrometastatic dissemination not found in conventional imaging or microscopic pathology frequently appeared. Considering the clinical course of most patients undergoing complete surgical resection, it is necessary to design new treatment strategies to suppress the regrowth of residual cancer and control the microdissemination of residual malignant cells after surgery. The first necessary step for this new strategy is developing an animal model for pancreatic cancer recurrence.

In this study, we validated a recurrence model following pancreatic cancer surgery and identified the most efficient time for detecting recurrence, which could serve as a basis for developing various treatments to prevent future recurrences. To establish this recurrence model, we utilized the AsPC-1 cell line renowned for its highly aggressive metastatic potential among available pancreatic cancer cell lines. Additionally, compared to other cell lines, it exhibits the most rapid growth rate and undergoes significant changes in vascular structure. Observable physical changes in tumor composition are also evident. Notably, this cell line shows high levels of vascular endothelial growth factor and epidermal growth factor. Furthermore, it grew to become poorly or moderately differentiated adenocarcinomas, closely resembling characteristics of tumors found in pancreatic cancer patients in clinical practice [10,15].

One of the most classic preclinical models for studying therapeutic strategies in cancer is a model prepared by injecting human tumor cells into an immunodeficient mouse, such as an immunocompromised nude mouse. Direct injection of tumor cells orthotopically into the pancreas of an immunodeficient mouse can create an ideal xenograft model that closely mimics the environment in which cancer cells grow and migrate. However, one disadvantage of this method is the potential for tumor cells to leak during the injection process. As an alternative to prevent cell leakage, an orthotopic xenograft model has been developed, in which tumor masses or Matrigel-tumor cell mixtures are directly transplanted into the pancreas of mice. It has been reported that these models can outperform other models in efficiently growing tumors by reducing cell

leakage [16]. However, in light of the importance of the tumor environment for tumor growth and progression, these models are less attractive for representing recurrence models after pancreatic cancer surgery. Therefore, we adopted the orthotopic cell injection method. Although this might seem trivial in an effort to prevent cell leakage, it is absolutely essential to minimize cell leakage by holding the syringe on the pancreas for 30–60 seconds after injection and inspecting the injection site while taking care not to perforate the thin dorsal side of the pancreas. In our mouse experiment, although not described in our results, cell leakage was found in only 5 of a total of 135 mice, accounting for a very small percentage of 3.7%.

Through our preliminary studies, the timing of tumor formation without metastasis was examined to determine the optimal timing for distal pancreatic resection. This process has indeed involved numerous trials and errors. We closely inspected the skin surface of each nude mouse and took weekly luminescence images after tumor cell injection to identify the specific time point at which tumors would reach their maximum sizes without invading surrounding tissues. Mice were divided into 4 groups (20 mice for each group). Complete resection was performed at 2, 3, 4, and 5 weeks after cell injection. During diagnostic laparotomy, tumors were observed to develop in the distal pancreas in 10 mice (50%) in the 2-week group and 16 mice (80%) in the 3-week group, showing no metastasis to surrounding organs. In 4-week and 5-week groups, over half of the mice exhibited tumor formation that had spread to surrounding organs. Therefore, we determined that the optimal timing for surgery after orthotopic injection was 3 weeks. Indeed, the choice of cell type, cell count, cell concentration, and the cell injection method, among other factors considered in the study, can significantly impact results, potentially revealing subtle variations. Consequently, the need for multiple adjustments and refinements was unavoidable.

When establishing a pancreatic cancer recurrence model, we observed sham and metastasis groups in addition to the resection group. These groups are essential experimental components to gain a comprehensive understanding of the disease and its progression. The sham model was designed to consider the potential effects of the surgery itself, such as surgical trauma, inflammation, stress response, and wound healing, which could influence tumor progression. By subjecting a sham group to the same procedure without primary resection or intervention, researchers can effectively identify and describe the specific impact of surgery on tumor recurrence. Metastasis groups can provide insight into distinct processes of tumor metastasis growth. This can help us develop targeted therapies to prevent or inhibit metastatic growth by understanding differences in tumor behavior between resection and metastatic groups. Therefore, by comparing sham, metastasis, and resection groups, researchers can analyze and understand distinct contributions of tumor growth, surgical effects, and metastasis. This approach not only advances our understanding of the disease but also facilitates the development of targeted therapies and treatments to address specific aspects of cancer progression. In our experiment, survival periods for sham and metastasis groups of mice were similar (33.1 and 35.1 days, respectively), while the resection group survived longer at 46.5 days. These results indicate that surgical treatment has a positive effect in improving the survival period of patients with pancreatic cancer.

Although surgical resection to treat pancreatic cancer allows complete removal of the main lesion, the resulting surgical trauma can cause an acute inflammatory reaction. The host's inflammatory response is rapidly stimulated due to surgical injury, causing release of cytokines, growth factors, and other chemical messengers. While these factors are essential for wound recovery, they have also been shown to play a significant role in cancer growth and metastasis [17,18]. Adjuvant chemotherapy is the most established treatment for eliminating residual pancreatic cancer cells and reducing the risk of recurrence. Patients usually have a recovery period of approximately 6 to 12 weeks after surgery. It is advisable to begin adjuvant chemotherapy within 8 weeks of the surgical procedure [19]. However, it has been well-documented that the occurrence of postoperative complications ranges from 30% to 60% [20]. When these complications do arise, they often result in a prolonged recovery period, potentially causing delays in subsequent treatments. Hence, it is crucial to establish a comprehensive treatment plan aimed at healing wounds and preventing pancreatic cancer recurrence throughout the recovery period. In recent times, there has been a surge in research into novel approaches including immunotherapy, targeted therapy for macrophages or growth factors, cancer vaccines, and dietary interventions like a ketogenic diet [19,21]. Our nude mouse model is anticipated to play a significant role in facilitating studies on the mechanisms and effects of these treatments.

In this study, the orthotopic model successfully gave shape to tumor recurrence, making it particularly useful for studying mechanisms of tumor regrowth and developing treatments after curative resection. Tumor growth was monitored in real-time and in a non-invasive manner using an *ex-vivo* imaging system in this model. This approach proved to be very useful in evaluating the effectiveness of the model. It will help us monitor the efficacies of anti-cancer drugs.

In conclusion, an orthotopic mouse model for pancreatic cancer recurrence after grossly complete resection was successfully developed. This model will be useful for studying tumor recurrence and metastasis after surgery as well as for developing new postoperative therapies.

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Conflict of Interest
No potential conflict of interest relevant to this article was reported.

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