Establishment and characterization of 6 novel patient-derived primary pancreatic ductal adenocarcinoma cell lines from Korean pancreatic cancer patients

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Abstract

Background: Pancreatic ductal adenocarcinomas are among the most malignant neoplasms and have very poor prognosis. Our understanding of various cancers has recently improved the survival of patients with cancer, except for pancreatic cancers. Establishment of primary cancer cell lines of pancreatic ductal adenocarcinomas will be useful for understanding the molecular mechanisms of this disease.

Methods: Eighty-one surgically resected pancreatic ductal adenocarcinomas were collected. Six novel pancreatic cancer cell lines, AMCPAC01–06, were established and histogenetic characteristics were compared with their matched tissues. The clinicopathologic and molecular characteristics of the cell lines were investigated by KRAS and TP53 sequencing or SMAD4 and p53 immunohistochemistry. Xenografts using AMCPAC cell lines were established.

Results: From the 81 pancreatic ductal adenocarcinomas, six (7.4% success rate) patient-derived primary cell lines were established. The six AMCPAC cell lines showed various morphologies and exhibited a wide range of doubling times. AMCPAC cell lines contained mutant KRAS in codons 12, 13, or 61 and TP53 in exon 5 as well as showed aberrant p53 (5 overexpression and 1 total loss) or DPC4 (all 6 intact) expression. AMCPAC cell lines demonstrated homology for the KRAS mutation and p53 expression compared with matched primary cancer tissues, but showed heterogeneous DPC4 expression patterns.

Conclusions: The novel AMCPAC01–06 cell lines established in this study may contribute to the understanding of pancreatic ductal adenocarcinomas.

Trial registration Retrospectively registered

Keywords: Pancreas, Cancer, Ductal adenocarcinoma, Primary, Cell line
Background
Ductal adenocarcinomas are the most common malignant neoplasms of the pancreas and account for approximately 85–90% of malignant neoplasms arising from the pancreas (known as pancreatic cancers). Pancreatic cancers are the ninth most common cancer in Korea [1], and the 5-year survival rate of patients with this type of cancer is only 9% [1]. Surgical resection is the mainstay for treating pancreatic cancer, but most patients are inoperable at the time of diagnosis, and only 30% of patients can undergo surgical resection [2]. Approximately 80% of patients show local recurrence or distant metastasis after surgical removal of tumors [2]. Other treatments, such as chemotherapies, are required to improve the survival time of patients with pancreatic cancer [3–6]. However, currently used chemotherapeutic regimens fail to significantly improve the survival time of these patients [7–9]. Therefore, development of new therapeutic modalities is important for improving the survival time of pancreatic cancer patients.

Recently, several trials were conducted to examine customized cancer treatment using patient-derived pancreatic cancer tissues [10–13]. However, using surgically resected pancreatic cancer tissue is difficult because of the limited amount of residual pancreatic cancer tissues after the submission of large amount of cancer tissues for pathologic examination for precise diagnosis and staging. To overcome this limitation, researchers have attempted to establish cancer cell lines from patient-derived cancer tissues and use various molecular pathologic studies with these cancer cell lines for tailored patient treatments. However, it is difficult to establish patient-derived primary cancer cell lines from pancreatic cancers because of specific histopathologic characteristics of pancreatic cancer such as low cancer cellularity of pancreatic cancer, and the occurrence of extensive desmoplastic reactions by overproduction of cancer-associated fibroblasts. Thus, the number of established patient-derived pancreatic cancer cell lines is currently much lower than that of cancers from other organs. At present, 21 pancreatic cancer cell lines have been established, including 11 cell lines from American Type Culture Collection and 3 cell lines from Korean Cell Line Bank [14–16].

In addition, some different clinicopathologic features were observed in pancreatic cancer patients of different ethnicities. Therefore, establishing novel primary pancreatic cancer cell lines derived from Korean pancreatic cancer patients and applying various chemotherapeutic regimens to the developed primary pancreatic cancer cell lines can help in the determination of highly sensitive chemotherapeutic regimens for individual pancreatic cancer patients, particularly when regional recurrence or distant metastasis develops.

Methods
Specimen collection
After approval (2015-0480) from the institutional review board, fresh tumor tissues measuring 0.5 × 0.5 × 0.2 cm³ in size were obtained from 81 surgically resected pancreatic ductal adenocarcinomas, immediately soaked in RPMI1640 media (Sigma-Aldrich Corp., St. Louis, MO, USA), and transferred in a laminar flow biosafety cabinet.

In vitro cell culture
Pancreatic tumor tissues were rinsed with Hank’s balanced salt solution in clean bench, minced into <2 mm³ fragments, and digested with 0.1% (W/V) of collagenase type 1 (GIBCO, Grand Island, NY, USA) at 37 °C for 10–20 min. The resulting fragments were centrifuged at 200×g for 5 min, washed thrice with phosphate-buffered saline, plated onto RPMI1640 media (GIBCO) containing 10% fetal bovine serum (GIBCO) and 1% penicillin/streptomycin (GIBCO), and allowed to adhere. After incubation for several days, mixed growth of cancer cells and fibroblasts was observed in the tissue fragments. To overcome fibroblast overgrowth, periodic tryspinization was conducted by incubation with 0.005% trypsin/EDTA (GIBCO) at 37 °C for 3 min during 2–3 passages to remove fibroblasts, and unwanted fibroblasts were detached by pipetting. The primary cell culture was monitored with a phase-contrast microscope. Cancer cells were grown at 37 °C in a humidified atmosphere with 5% CO₂.

Growth rate analysis of established cell lines
The cell growth rate was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) at 24-h intervals. After 1 × 10⁴ cells were seeded into 96-well plates, 0.5 mg/mL MTT was added over consecutive days for violet pellet formation by living cells. The pellets were solubilized in 200 μL of dimethyl sulfoxide. The optical density of each sample was measured at 570 nm using a microplate reader (Sunrise Reader, Tecan, Männedorf, Switzerland). Growth rate was measured as a percentage of control growth. Cells from passage 15 were used to determine population doubling time, and all experiments were repeated twice in triplicate.

Characterization of cell lines
Construction cell microarray
After fixation of 5 × 10⁶ cancer cells with a Cytorich Red fixative solution (BD Biosciences, Franklin Lakes, NJ, USA) for 48 h, the supernatant was removed after centrifugation. The pellets were additionally fixed with 95% ethanol for 60 min then embedded in paraffin. Each cancer cell block was selected as a donor, and the
designated areas for each cell block were punched with a 5-mm diameter cylinder by a Manual Tissue Microarrayer (Uni TMA Co., Ltd., Seoul, Korea) and transferred to a recipient block, and cell microarrays (CMAs) were constructed.

Immunohistochemistry
Immunohistochemical labeling was performed by the immunohistochemical laboratory of the Department of Pathology, Asan Medical Center. Briefly, 4-μm tissue sections from the CMA and matched formalin-fixed paraffin-embedded (FFPE) primary cancer tissues of ductal adenocarcinomas were deparaffinized and hydrated in xylene and serially diluted with ethanol, respectively. Endogenous peroxidase was blocked by incubation in 3% H₂O₂ for 10 min, and heat-induced antigen retrieval was performed. Primary antibodies with Benchmark autostainer (Ventana Medical Systems, Tucson, AZ, USA) were used as per the manufacturer’s protocol. Primary antibodies for cytokeratin 19 (clone A53-B/A2.26; 1:200; Cell Marque, CA, USA), p53 (clone DO-7; 1:3000; DAKO, Glostrup, Denmark), and DPC4 (clone EP618Y, 1:100; Genetex, Irvine, CA, USA) were incubated at room temperature for 32 min, and the sections were labeled with an automated immunostaining system with the I-View detection kit (Benchmark XT; Ventana Medical Systems). Immunostained sections were lightly counterstained with hematoxylin, dehydrated in ethanol, and cleared in xylene.

Detection of TP53 and KRAS mutations
The genomic DNA of the established cell lines was extracted using the QIAamp DNA Micro kit (Qiagen, Hilden, Germany) following the manufacturer’s protocol. Polymerase chain reaction (PCR) amplification was performed with 10 ng of DNA covering exons 5–8 of the TP53 gene with intragenic primers flanking these exons as previously described [17]. PCR-amplified products were purified using a QIAquick column (Qiagen). TP53 gene sequencing was performed with BigDye 3.1 and a 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA).

Similarly, pyrosequencing was performed to detect KRAS at codons 12, 13, and 61. Primer sequences of KRAS are summarized in Table 1. DNA (10 ng) was amplified using a biotin-labeled primer covering codons 12, 13, and 61 of KRAS. The biotin-labeled PCR products were immobilized on Streptavidin Sepharose HP beads (GE Healthcare, Little Chalfont, UK) and the immobilized PCR products were sequenced using a pyrosequencer, PyroMark Q96MD System (Qiagen) according to the manufacturer’s protocol. Primer sequences used to amplify TP53 and KRAS are shown in Table 1.

In vivo tumorigenicity by tumor xenograft
Tumorigenicity in mice was confirmed by subcutaneous injection of approximately 2 × 10⁶ cancer cells from culture bilaterally into each flank of male NSG (5 weeks old) mice. Xenograft tumor growth was monitored twice per week for 3 months. Tumor volume (TV) was calculated according to the formula: TV (mm³) = length × width² × 0.5. When a tumor size of 100–200 mm³ was reached, tumors were explanted from the mice. The explanted tumor xenografts were used for reimplantation and FFPE to confirm the histology.

Statistical analysis
The significance of differences between experimental conditions was determined using the student’s t test and Mann–Whitney U test for unpaired observations.

Results
Establishment of pancreatic cancer cell lines
Fresh tissues from 81 patients diagnosed with pancreatic ductal adenocarcinomas were used to establish primary cancer cell lines. Among the 81 cases, 75 cases (93%) failed to establish cell lines because of fungal or mycoplasmal contamination in 30 cases (37%) and overgrowth of cancer-associated fibroblasts in 45 cases (56%). Six (7.4%) patient-derived pancreatic cancer cell lines were

| Target    | Forward primer | Reverse primer | AT (°C) | Size (bp) |
|-----------|----------------|----------------|---------|-----------|
| TP53 exon 5 | 5'-CACTTGTGCCCCGTACTTCC-3' | 5'-AACCAAGCCCCTGTCGCTCT-3' | 64      | 267       |
| TP53 exon 6 | 5'-CAGGCCCTCTGATCTCCTACT-3' | 5'-CTTAACCCCTCCTCCAGAG-3' | 64      | 185       |
| TP53 exon 7 | 5'-CCACAGTGGCTCCTCAAGG-3' | 5'-CCAGGTGCAAGCGACCTT-3' | 64      | 179       |
| TP53 exon 8 | 5'-GCTCCTTCTTCTCTTTC-3' | 5'-TAATTCGCCGCGCTCCT-3' | 62      | 217       |
| KRAS 12 and 13 | 5'-GGTGAGTTTGTATTAAAGGTACCTG-3' | 5'-Biotin-GCTGTATCGTCAAGGCACTCTT-3' | 56 | 100       |
| KRAS 61 | 5'-TGGAGAAAAACCTGTCCTGATAT-3' | 5'-Biotin-TACTGGGCACTCATTGCACTGTA-3' | 60 | 72        |

AT: annealing temperature
established and named, AMCPAC01–AMCPAC06. The first sub-culture was performed 7–15 days after initiating primary culture for attachment and spreading, and the AMCPAC cell lines were passed a minimum of 10 times.

Clinicopathologic characteristics of cancer cell lines
Clinicopathologic characteristics of the established cancer cell lines are summarized in Table 2. Briefly, cell lines were derived from five males and one female. The mean age of the patients was 52.0 ± 12.1 years (range 30–67 years). The pathologic diagnosis of all six cases was moderately differentiated ductal adenocarcinoma (Fig. 1). Tumor locations were as follows: tail of the pancreas in four cases, body in 1 case, and head in 1 case. The mean tumor size was 3.6 ± 1.8 cm (range 2.1–6.9 cm). Five cases showed peripancreatic soft tissue extension, while one case invaded the surrounding organs, spleen, and transverse colon (AMCPAC04). Lymphovascular

| Clinicopathologic factors          | AMCPAC01 | AMCPAC02 | AMCPAC03 | AMCPAC04 | AMCPAC05 | AMCPAC06 |
|-----------------------------------|----------|----------|----------|----------|----------|----------|
| Age (years)                       | 30       | 67       | 56       | 53       | 52       | 54       |
| Sex                               | Male     | Female   | Male     | Male     | Male     | Male     |
| Operation name                    | DP       | DP       | DP       | DP       | PPPD     | DP       |
| Pathologic diagnosis              | Ductal adenocarcinoma | Ductal adenocarcinoma | Ductal adenocarcinoma | Ductal adenocarcinoma | Ductal adenocarcinoma | Ductal adenocarcinoma |
| Differentiation                    | MD       | MD       | MD       | MD       | MD       | MD       |
| Location                          | Body     | Tail     | Tail     | Tail     | Head     | Tail     |
| Tumor size (cm)                   | 2.1      | 2.5      | 4.5      | 6.9      | 2.8      | 2.9      |
| pT classification                 | pT3      | pT3      | pT3      | pT4      | pT3      | pT3      |
| pN classification                 | pN0      | pN0      | pN1      | pN1      | pN0      | pN1      |
| Lymphovascular invasion           | Absent   | Present  | Absent   | Present  | Present  | Absent   |
| Perineural invasion               | Present  | Present  | Present  | Present  | Present  | Absent   |

DP distal pancreatectomy, PPPD pylorus preserving pancreatecticioduodenectomy, MD moderately differentiated

Fig. 1 Representative hematoxylin and eosin staining images of FFPE pancreatic cancer tissue
invasions were identified in 3 cases (AMCPAC02, AMCPAC04, and AMCPAC05) and perineural invasions were observed in 5 cases, except for case #6. Lymph node metastases were detected in 3 cases (AMCPAC03, AMCPAC04, and AMCPAC06).

**Cytological characteristics of cancer cells**

To more efficiently investigate cytological characteristics, CMAs were constructed with the AMCPAC cell lines. The CMA block was composed of 6 wells containing each AMCPAC cell line, and CMA sections were stained with hematoxylin and eosin; matched tissue samples of pancreatic ductal adenocarcinomas were included (Fig. 2b). Cytological evaluation revealed that all six cancer cell lines were adenocarcinomas with mucin production and monolayer growth on the culture dish surface (Fig. 2a). Cytologically, all 6 cancer cell lines showed round to oval

![Fig. 2](representative AMCPAC cell line images. a Morphology of AMCPAC01–AMCPAC06 cell lines. b H&E staining images of AMCPAC01–AMCPAC06 (all, ×20)]
and AMCPAC06) or polygonal (AMCPAC04) shapes. The population doubling time of cancer cells was measured by MTT assay. The growth rates of AMCPAC04 and AMCPAC06 were rapid (doubling time of approximately 2 days), while those of AMCPAC02, AMCPAC03, and AMCPAC05 were relatively slow (approximately 3–4 days) based on 24-h growth after cell seeding (Table 3; Fig. 3).

**Table 3**  Cytologic characteristics and growth rate of established cell lines

| Cell line   | Growth characteristics | Cell morphology | Population doubling time (h) |
|-------------|------------------------|-----------------|------------------------------|
| AMCPAC01    | Adherent               | Round/oval      | 68                           |
| AMCPAC02    | Adherent               | Round/oval      | 87                           |
| AMCPAC03    | Adherent               | Round/oval      | 68                           |
| AMCPAC04    | Adherent               | Polygonal       | 48                           |
| AMCPAC05    | Adherent               | Round/oval      | 75                           |
| AMCPAC06    | Adherent               | Round/oval      | 51                           |

Mutational and immunohistochemical status of cancer cell lines

Mutant *KRAS* was detected in all AMCPAC cell lines. Mutations in *KRAS* codon 12 were detected in AMCPAC01, AMCPAC02, AMCPAC03, and AMCPAC06, while a mutation in *KRAS* codon 13 was detected in AMCPAC04. And two mutations in *KRAS* codons 60 and 61 were detected in AMCPAC05 (Table 4). Representative images of *KRAS* mutations are depicted in Fig. 4.

Sequencing analysis of *TP53* revealed the deletion of exon 5 in AMPCPAC01 and missense mutations in 3 lines (AMCPAC04, AMCPAC05, and AMCPAC06) based on Clinvar database analysis (Table 5, Additional file 1: Figure S1).

Representative images of DPC4 and p53 immunolabeling are shown in Figs. 5 and 6 and summarized in Table 6. All 6 cancer cell lines showed intact DPC4 labeling (Fig. 5a). Overexpression of p53 protein was observed in 5 cancer cell lines (AMCPAC02–06; Fig. 6a), while 1 cell line showed a total loss of p53 expression (AMCPAC01). All 6 cases showed matched p53 expression patterns between pancreatic cancer tissue and primary cell lines (Fig. 6b). However, only AMCPAC04 showed matched DPC4 expression between the primary tumor and cancer cell line, while the other 5 tissues showed heterogeneous expression of DPC4 (Fig. 5b).

**Table 4**  *KRAS* mutation analysis in AMCPAC cell lines

| Cell line   | c. Description | Codon number | Protein description | Mutation type |
|-------------|----------------|--------------|---------------------|---------------|
| AMCPAC01    | c.35_36GT>TC^a | 12           | p.Gly12Val(G12V)    | Missense      |
| AMCPAC02    | c.35G>A^a      | 12           | p.Gly12Asp(G12D)    | Missense      |
| AMCPAC03    | c.35G>A^a      | 12           | p.Gly12Asp(G12D)    | Missense      |
| AMCPAC04    | c.38G>A^a      | 13           | p.Gly13Asp(G13D)    | Missense      |
| AMCPAC05    | c.180_181insCTA| 60, 61       | p.Gly60_Gln61insLeu | Insertion     |
| AMCPAC05    | c.182A>T^a     | 61           | p.Gln61Leu(Q61L)    | Missense      |
| AMCPAC06    | c.34G>C^a      | 12           | p.Gly12Arg(G12R)    | Missense      |

NA not applicable

* Pathogenic
Table 5  Exon 5 on TP53 mutation in AMCPAC cell lines

| Cell line | c. Description | Codon number | Protein description | Mutation type |
|-----------|----------------|--------------|---------------------|---------------|
| AMCPAC01  | c.384_393del10  | 128–131      | N.D deletion         |               |
| AMCPAC02  | N.A            | N.A          | N.A wild-type       |               |
| AMCPAC03  | N.A            | N.A          | N.A wild-type       |               |
| AMCPAC04  | c.380C>Tb      | 127          | p.S127F missense    |               |
| AMCPAC05  | c.398T>A       | 133          | p.M133K missense    |               |
| AMCPAC06  | c.451C>Tb      | 151          | p.P151S missense    |               |

NA not applicable, N.D not detectable

a Likely pathogenic
b Pathogenic
Fig. 5  Heterogeneous DPC4 expression within original cancer tissues compared with AMCPAC cell line.  

**a** DPC4 expression in AMCPAC cell lines.  

**b** DPC4 expression in cancer tissues
It is known that KRAS, p16/DCKN2A, GNAS, and BRAF are mutated early in pancreatic cancer progression, while SMAD4/DPC4 and TP53 are mutated at later stages [25, 46]. To compare DPC4 and p53 expression levels between established cell lines and matched cancer tissues, immunocytochemistry was performed on CMA sections containing 6 AMCPAC cell lines and matched cancer tissues. Five cell lines, AMCPAC02-06, and matched cancer tissues showed p53 protein overexpression, while AMCPAC01 showed a total loss of p53 expression. A lack of p53 expression is associated with a nonsense mutation (or null mutation) in TP53 [47]. Our TP53 sequencing revealed a deletion (TGCCCTCAAC) on exon 5 of TP53 which was associated with the loss of p53 protein expression. Several studies reported worse prognosis of cancer patients with a lack of p53 expression.
Table 6  Summary of DPC4 and p53 immunohistochemistry staining in AMCPAC cell lines and matched cancer tissues

| Cell line   | Primary cancer cell line | DPC4   | P53      | Cancer tissue                                  |
|-------------|--------------------------|--------|----------|-----------------------------------------------|
| AMCPAC01    | Intact                   | Total loss |          | Heterogeneous (intact/loss)                  |
| AMCPAC02    | Intact                   | Overexpression |      | Total loss                                  |
| AMCPAC03    | Intact                   | Overexpression |      | Overexpression                              |
| AMCPAC04    | Intact                   | Overexpression |      | Overexpression                              |
| AMCPAC05    | Intact                   | Overexpression |      | Overexpression                              |
| AMCPAC06    | Intact                   | Overexpression |      | Overexpression                              |

Fig. 7  Construction of xenograft of AMCPAC cell lines
compared to those with missense TP53 mutations in lung and ovarian cancers [48, 49].

Because DPC4 protein loss is correlated with SMAD4/DPC4 mutation, immunohistochemical staining for DPC4 protein is used as a diagnostic criteria of pancreatic cancer with lower cost than sequencing of SMAD4/DPC4 [50]. Loss of DPC4 expression is associated with distant metastasis, epithelial to mesenchymal transition, and treatment failure to local recurrence [51–53]. In the present study, all 6 AMCPAC cell lines showed homogeneously intact DPC4 expression. In contrast, matched cancer tissues showed heterogeneous DPC4 expression: one tumor area exhibited intact DPC4 expression, while another region showed loss of DPC4 protein expression (Fig. 8). Homogeneously intact DPC4 expression in the primary pancreatic cancer cell lines, which were obtained from cancer tissues with intratumoral DPC4 heterogeneity, can be explained by the positive selection of DPC4-expressing cancer cells during primary cell culture or most DPC4 expressing cancer cells presented in cancer tissues specimens at the time of primary cell culture (Fig. 8).

Genetic heterogeneity occurs in various situations, such as between separate cancers with the same histologic subtypes, primary and metastatic cancers from the same individuals, and separate regions from the same cancers [54–58]. Additionally, genetic heterogeneity has been observed in many solid tumors, including renal cell carcinomas, non-small cell lung cancers, and breast cancers [54, 55, 59–61]. The organoid model of pancreatic cancer was recently examined among preclinical models for predicting personalized tailored therapy [22–24]. This model is expected to conserve the tumor microenvironment, including ductal or acinar structures, and reflect tumor heterogeneity. Therefore, to understand interactions in tumor microenvironments or develop

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**Fig. 8** Original cancer tissue of AMCPAC04 showed heterogeneous DPC4 expression. A Low-power scanning view shows heterogeneous DPC4 expression (magnification, ×40). B Higher power view shows area of loss of DPC4 expression (×200). C Cancer cells on the left half show intact DPC labeling, while cancer cells on the right half show loss of DPC4 expression (×100). D Higher power view shows area of intact DPC4 expression (×200). E Cancer cells in AMCPAC04 cell line show intact DCP4 labeling (×200).
personalized medicine, application of the organoid model will be more effective than conventional primary cancer cell cultures. However, the traditional primary cancer cell culture model is more suitable for investigating the characteristics of pure cancer cells. Nevertheless, methods for establishing specific cell lines by modifying organic models and our culture protocols should be examined in future studies.

AMCPAC cell lines can provide a resource for pancreatic cancer studies, including the basic and translational research fields. For example, comparison of DPC4(+) / P53(−) (AMCPAC01) and DPC4(+) / P53(+) (AMCPAC02–06) cell lines will be helpful in understanding the interactions of TP53 and DPC4 signaling pathways. In total, 3 cell lines have been previously established from Korean pancreatic cancer patients, including SNU213, SNU324, and SNU410, which are available from the Korean Cell Line Bank. We have added 6 newly established pancreatic cancer cell lines from Korean patients. Different molecular pathologic characteristics of our new established AMCPAC cell lines may provide diversity and help in determining the molecular pathological basis of pancreatic cancer in different ethnicities from other previously established pancreatic cancer cell lines.

Conclusions
We established and characterized 6 novel pancreatic cancer cell lines (AMCPAC01–AMCPAC06). These novel cell lines may contribute to the understanding of pancreatic ductal adenocarcinomas, including the molecular basis of tumorigenesis, progression, and metastasis of pancreatic cancers.

Additional file

Additional file 1: Figure S1, TP53 histogram of AMCPAC cell lines.

Abbreviations
CMA: cell microarray; FFPE: formalin-fixed paraffin-embedded.

Authors’ contributions
SJK, SYA, HSP, JHL, KBS, DWH, and SCK collected patient tumor tissue samples to establish cell lines and analyzed clinical information. MJK and MSK performed experimental work. JP implanted xenografts into mice. MJK, SYJ, KPK, SCK, and SMH conceived ideas and evaluated the manuscript. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate
The study protocol was approved by the institutional review board (IRB) of the Asan Medical Center (2015-0480). The animal care protocol for this study was approved by the International Animal Care and Use Committee (IACUC) of the Laboratory of Animal Research at the Asan Medical Center, Seoul, Korea.

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