Generation and genome analysis of patient-derived pancreatic cancer cell line using conditionally reprogrammed cell method

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Generation and genome analysis of patient-derived pancreatic cancer cell line using conditionally reprogrammed cell method

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ABSTRACT

Generation and genome analysis of patient-derived pancreatic cancer cell line using conditionally reprogrammed cell method

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(Directed by Professor Seungmin Bang)

INTRODUCTION: Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal malignancy. One of the greatest challenges in PDAC research has been the generation of stable cancer cell lines from primary tumors. Conditionally reprogrammed cell (CRC) technology is a novel cell-culture system enabling the generation of stable cell cultures. CRCs can be grown indefinitely under defined conditions without the use of genetic immortalization techniques. The objective of this study was to utilize the CRC technology in human PDAC specimens to develop cancer cell lines that phenotypically represent native tumors.

METHODS: We obtained the tumor specimens from resection specimens

for patients with operable PDAC and from endoscopic ultrasound-guided biopsy or percutaneous liver biopsy for patients with inoperable PDAC. Cancer cells were co-cultured with irradiated feeder cells and the Rho-associated kinase inhibitor Y-27632 to develop CRCs. To enable the rational design and testing of patient-origin cancer cell lines for patients with PDAC, we analyzed the CRCs at the genetic level by whole exome sequencing. *In vivo*, NOD/SCID mice (5-week-old male) were injected with CRCs (2×10^6) in the right and left flank. After sacrifice, the tumor size was measured and implanted tumor tissue was fixed in paraformaldehyde for histologic analysis.

RESULTS: Sixteen (34.8%) CRCs were established from 46 PDAC patients. We confirmed that the genetic characteristics of cancer tissues were preserved in CRCs by comparing the AFs of somatic mutations found in cancer tissues and CRCs. Mutation profiles were 100% concordant between original PDAC tissue and CRCs for 3 patients with PDAC. *In vivo*, 3 CRCs were implanted in NOD/SCID mice, and routine hematoxylin and eosin histology revealed that the implanted tumor tissue showed identical morphology to the parent tumor tissue. The allele frequency of key oncogenic mutations common to both tumor and CRCs, including TP53, SMAD4, and KRAS, was either higher in CRCs or

similar in both groups.

CONCLUSIONS: We established the first PDAC cell line system to represent original PDAC tissue. The ability to rapidly generate patient-origin cancer cells from small tumor specimens would facilitate the development of individualized treatment and could be used to identify effective drug combinations for PDAC.

Key words: Pancreatic ductal adenocarcinoma, Conditional reprogramming cells, Next-generation sequencing, Patient-derived cancer cell line

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Pancreatic ductal adenocarcinoma (PDAC) has the worst prognosis of all cancers, with an overall 5-year survival of approximately 6%, and it is expected to become the second leading cause of cancer deaths by 2030.[1] Even in patients who undergo complete resection, long-term survival is poor owing to tumor recurrence.[2]

Recently, high-throughput next-generation sequencing studies have provided significant insights into the genomic landscape of several tumor types, revealing molecularly defined tumor subtypes, identifying new druggable targets, and shedding light on to the heterogeneity of many tumors.[3] However, unlike other cancers, the number of sequenced PDAC genomes is relatively modest. This fact reflects the difficulty of sequencing a tumor with a predominance of stromal and inflammatory cells and from which it is difficult to obtain sufficient tissue specimens.[4] Therefore, patient-specific cancer cell lines must be established to investigate these molecular events and predict chemotherapy

responsiveness. To generate stable cancer cell lines from primary PDAC, many models have been developed recently. However, their stability over time and relationship to the primary cancer in many cases are marginal. Additionally, most primary cell cultures suffer from a limited lifespan because of cellular senescence.[4,5]

Recently, the Schlegel group at Georgetown developed a new approach known as "conditional reprogrammed cells" (CRCs).[6] They showed that patient-derived tumor cells could be grown indefinitely under defined conditions without the use of extrinsic genetic immortalization techniques. These cells were shown to maintain a karyotype similar to the tissue of origin even after prolonged passaging. This technology enabled the growth of tumor cells from very small biopsy specimens.

In the present study, we aimed to develop PDAC cell lines that phenotypically represent primary human PDAC specimens using the CRC method. To identify whether the newly cultured cells were representative of the primary tumor, we conducted cancer genomic analysis and experimental verification.

II. MATERIALS AND METHODS

1. Patients and tissue samples

Patients who were diagnosed with PDAC were enrolled for establishment of the tumor model and genetic analysis. Tumor specimens (≤1 cm) were obtained from resected specimens of patients who underwent operation for PDAC. For patients with unresectable PDAC, endoscopic ultrasound (EUS)-guided biopsy or percutaneous biopsy were performed to obtain the tumor specimens. Tumor tissues and paired peripheral blood samples were collected simultaneously in the present study. All tissues were placed into medium with antibiotics. Using forceps and a scalpel, residual fat tissue was removed. Tumor tissues were minced into 1-2 mm small fragments with sterile scissors. Dissected specimens were placed in medium. Primary cell line isolation was conducted within 1–2 h of tumor resection. If specimens could not be processed immediately to prepare CRCs, the tumor cells were frozen in liquid nitrogen for long-term storage. Tissue was re-suspended in collagenase (1 mg/mL, Sigma, St. Louis, MO, USA) in culture medium and incubated for 30 min at 37℃ with agitation to dissociate the tumor tissue from the collagenous stroma. We added 5x F-medium for neutralization, followed by centrifugation at 1500 rpm for 3 min. The supernatant was filtered through a cell strainer (70-μm nylon, falcon). The filtered tumor cells were re-suspended in F-media consisting of Keratinocyte-SFM (Life Technologies, Carlsbad, CA, USA) supplemented with prequalified recombinant Epidermal Growth Factor and Bovine Pituitary

Extract (Life Technologies), 2% fetal bovine serum (Sigma), and 1% antibiotic-antimycotic (Life Technologies). The cells were cultured on plates pre-plated with irradiated 3T3-J2 (irradiated mouse fibroblast cells). Cells were incubated at 37℃ with 5% CO_2. Tumor cells on the plates were readily apparent by morphology relative to stromal elements (e.g. fibroblasts). Contaminating stromal cells were removed by differential trypsinization or selective scraping of the plates as necessary. The cell lines were pre-treated with 500 ng/mL mycoplasma removal agent (MP Biomedicals, Santa Ana, CA, USA). The cell lines generated were regularly checked to ensure that they were not infected with mycoplasma.

All patients signed consent forms for sample collection and molecular analysis. The study was approved by the institutional review board of Severance Hospital, Seoul, Korea. All experiments were performed in accordance with relevant guidelines.

2. CRC method

We used the CRC method developed by the Schlegel group at Georgetown. Tissue samples were co-cultured with J2 murine fibroblast feeder cells and medium containing the Rho-kinase inhibitor Y-27632 (Figure 1). Cells were seeded on a feeder layer of lethally irradiated (30 Gy) J2 fibroblasts in F medium. The F medium consisted of 70% Ham's F-12 nutrient mix (Hyclone,

Logan, UT, USA) and 25% complete Dulbecco's modified Eagle medium, supplemented with 0.4 μg/mL hydrocortisone (Sigma), 5 μg/mL insulin, 8.4 ng/mL cholera toxin (Sigma-Aldrich, St. Louis, MO), 10 ng/mL epidermal growth factor, 5% fetal bovine serum (Hyclone), 24 μg/mL adenine (Sigma), 10 μg/mL gentamycin (Life Technologies), and 250 ng/mL Amphotericin B (Thermo Fisher Scientific, Waltham, MA, USA). Cells were cultured in the presence of the Rho-associated kinase (ROCK) inhibitor, Y-27632 at a final concentration of 5 μM (Enzo Life Sciences, Farmingdale, NY, USA).

3. Cell characterization

A. Immunofluorescence assay

Cells were fixed in 4% paraformaldehyde for 15 min at room temperature (RT) and washed in PBS three times, followed by blocking with 5% normal goat serum for 1 h and incubation with primary antibodies at room temperature (1 h), washing three times in PBS, and incubation with second antibody for 30 min. The following primary antibodies were used: a-Amylase (A8273, rabbit polyclonal, dilution 1:100; Sigma), cytokeratin 19 (A53-B/A2: sc-6278, mouse monoclonal, dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), insulin (mouse monoclonal, dilution 1:100; Zymed, Waltham, MA, USA), and vimentin (V9: sc-6260, mouse monoclonal, dilution 1:100; Santa Cruz Biotechnology). The cells were stained with Alexa Flour conjugated secondary antibodies from Invitrogen.

B. Soft agar colony formation assay

To evaluate tumorigenicity, *in vitro* tumorigenesis was examined by soft agar culture. The anchorage-independent growth of CRCs was evaluated by colony formation assay in soft agar. Soft agar colony formation assays were performed using the double-layer soft agar method. In each well of a 6-well plate, 5×10^4 cells were plated on the top agar (0.5% agarose gel) over a base agar (1% agarose gel). After 2–3 weeks of incubation in soft agar, the average numbers of colonies formed by CRCs were checked.

C. KRAS mutation analysis by polymerase chain reaction (PCR)

KRAS mutations were analyzed by PCR. The methods used to establish KRAS mutation are essentially divided into sequencing and PCR based methods. Sequencing may lack sensitivity, particularly in the presence of large amounts of wild-type DNA from infiltrating cells, while PCR methods often show better sensitivity. Genetic analysis of the KRAS gene was performed by PCR amplification of exons 1 (codon 12 and 13), followed by direct sequencing of the PCR products. DNA was extracted using QIAGEN QIAamp® DNA Mini Kits (Hilden, Germany).

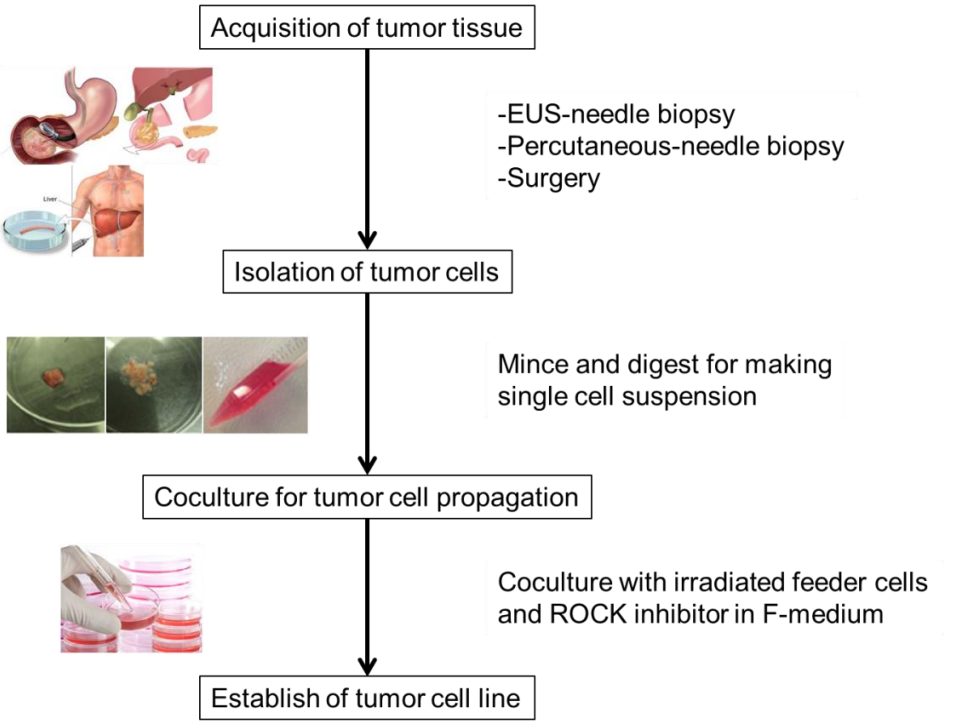

Figure 1. Process flow chart. PDAC cells obtained from surgically resected or biopsy tissue were grown with irradiated fibroblasts and ROCK inhibitor[7].

EUS, Endoscopic ultrasound; ROCK, Rho kinase

4. Targeted deep sequencing and preprocessing

We validated the suitability of established CRCs by comparing their 83 targeted gene sequences with those of original PDAC tissue to develop representative genomic data of PDAC (Table 1). In the first step, DNA obtained by microdissection of formalin-fixed paraffin-embedded tissue of PDAC and DNAs from pancreatic CRCs of the same patient were sequenced. DNA was extracted using the QIAamp DNA Mini Kit and sequenced with a HiSeq2500/Miseq (Illumina, San Diego, CA, USA). Quality control of the DNA was performed using a Nanodrop spectrophotometer (Thermo Fisher Scientific).

Preprocessing of read sequences were conducted by quality filtering using fastQC. We followed the Genome Analysis Toolkit (GATK) best practice for improving the quality of variant calls. Sequencing reads in targeted region were aligned and compared to the human reference genome (UCSC hg19) using BWA-MEM version 0.7.10 and Picard tools version 1.119 (http://broadinstitute.github.io/picard/). According to the GATK best practice, the location of insertions and deletions was recalibrated based on the dbSNP version 150 database of known variants. Cross-contamination of tumors and CRC samples was estimated using ContEst.

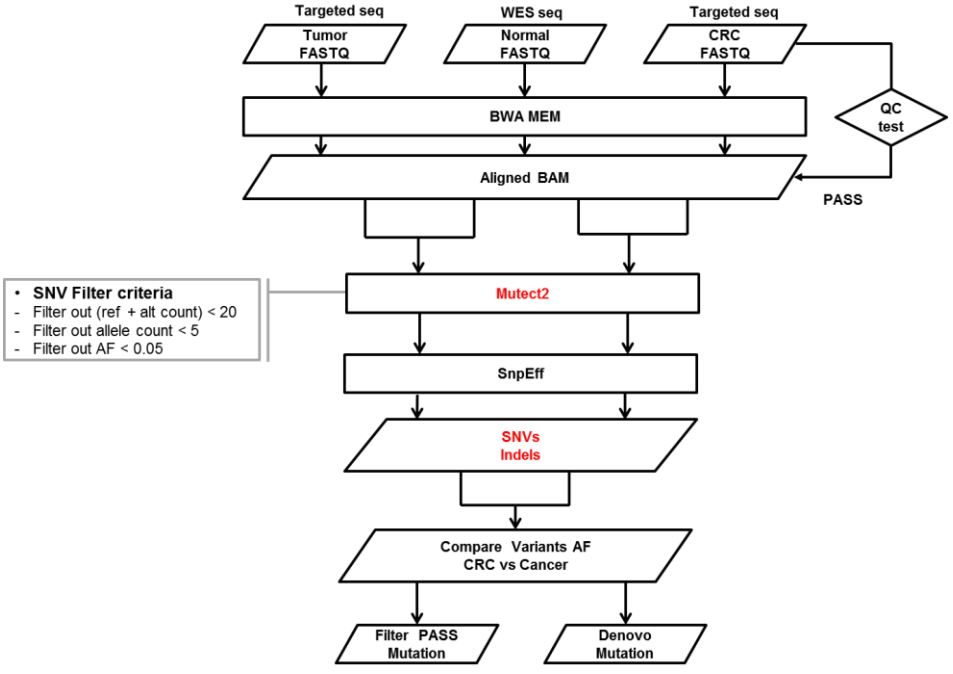

Figure 2. Sequencing flow chart and criteria for filtering single-nucleotide variants (SNVs). Read sequences were preprocessed by quality filtering using fastQC. Somatic SNVs and indels were identified in normal-tumor paired and normal-CRCs paired samples by GATK version 3.5 Mutect2. AF, Allele frequency; CRCs, Conditionally reprogrammed cells.

5. SNV and indel calling

Somatic single-nucleotide variants (SNVs) and indels were identified in normal-tumor paired and normal-CRC paired samples by GATK version 3.5 Mutect2. SNVs in 83 targeted genes were annotated with genetic features using SnpEff and SnpSift version 4.1 using dbSNP. Additionally, we annotated common somatic mutations in the Catalog of Somatic Mutation in Cancer database.

To retain high-confidence somatic variants with the Mutect2 filter, we applied the following filter criteria: 1) variants with alternate allele counts less than 5, 2) variants with total allele counts (read depth) less than 20, and 3) variants with allele frequency (AF) less than 0.05 (Figure 2). Detected variants were further manually confirmed using IGV viewer version 2.3.81 to reduce false-positive variants. A non-synonymous mutation in one patient's CRCs was defined as a *de novo* mutation when the alternate allele count of the patients' tumor was less than 5.

6. Copy number variant (CNV) calling

CNV calling was performed using CNVkit version 0.8.6.9. To reduce copy number biases, we combined all normal samples into a pooled reference to compare tumor samples. The corrected on- and off-target log2 value of bin-level copy ratios with associated weights were concatenated using the fix command. The call command with the center mode option was used to

recalculate the copy number of the default center-centering the copy number neutral area slightly above or below the expected log2 value of zero. After these adjustments, we used threshold methods to calculate the absolute integer copy number of each segment.

7. Establishment of patient-derived xenografts using CRCs

Exponentially growing PDAC CRCs were trypsinized, dispersed into single cells, and suspended in 200 μL of Matrigel HC (BD Biosciences). To evaluate *in vivo* tumorigenicity, 2×10^6 cells in 0.2 mL of Matrigel were injected subcutaneously into the bilateral flank regions of 5-week-old male NOD/SCID mice with severe combined immunodeficiency (Charles River Laboratories, Tokyo, Japan). Animals were housed at the Yonsei University animal care facility according to institutional guidelines. Monitoring for tumor growth was performed up to 6 months post-implantation. Tumor size was assessed by measurement with calipers once per week. The mice were sacrificed by CO_2 inhalation; tumors were harvested once they reached volume ~1500 mm^3 (20 mm). PDX tumor tissue was divided for: 1) fixation in 10% buffered formalin for paraffin embedding/histology, 2) cryopreservation, 3) PDX cell line development and passaging, and 4) optimal cutting temperature compound embedding (Figure 3).

8. Cell viability assay and drug sensitivity

A cell viability assay was performed to determine the drug sensitivity of each cell line. CRCs of patients with different prognoses were selected to test the response to gemcitabine. Cells were seeded at 1–3 × 10^3/well in 96-well plates and exposed to various concentrations of gemcitabine for 72 h. The effects of gemcitabine on cell viability were assessed by the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT; Amresco, Solon, OH, USA) assay. Values are the means of triplicate wells from three independent experiments for each drug concentration.

Table 1. Eighty-three targeted gene sequences

ABL1	BRCA2	FGFR2	KIT	PTCH1
AKT1	CDH1	FGFR3	KRAS	PTCH2
AKT2	CDK4	FLT3	MDM2	PTEN
AKT3	CDK6	GNA11	MET	PTPN11
ALK	CDKN2A	GNAQ	MLH1	RB1
APC	CSF1R	GNAS	MPL	RET
ARID1A	CTNNB1	HNF1A	MTOR	ROS1
ARID1B	DDR2	HRAS	NF1	SMAD4
ARID2	EGFR	IDH1	NOTCH1	SMARCB1
ATM	EPHB4	IDH2	NPM1	SMO
ATRX	ERBB2	IGF1R	NRAS	SRC
AURKA	ERBB3	ITK	NTRK1	STK11
AURKB	ERBB4	JAK1	PDGFRA	SYK
BCL2	EZH2	JAK2	PDGFRB	TOP1
BRAF	FBXW7	JAK3	PIK3CA	TP53
BRCA1	FGFR1	KDR	PIK3R1	VHL
EWSR1	TMPRSS2	TERT		

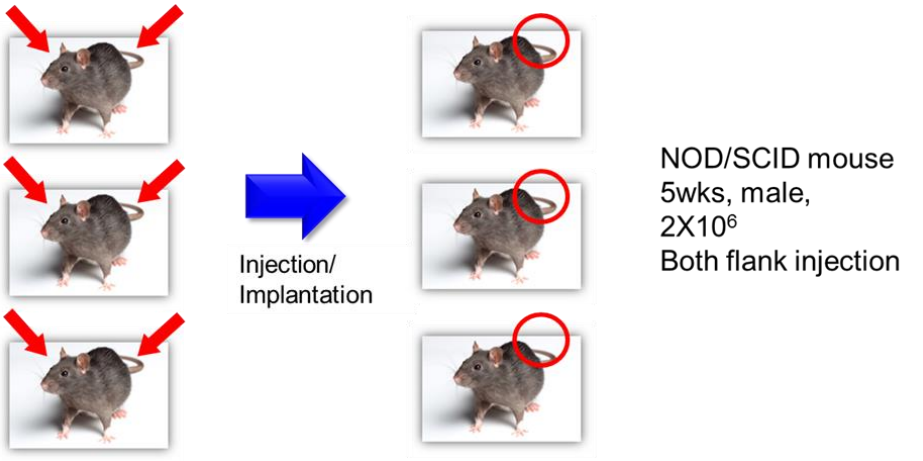

Figure 3. Establishment of patient-derived xenografts using CRCs.

A total of 2 × 10^6 CRCs in 0.2 mL of Matrigel were injected subcutaneously into the left and right flanks of 5-week-old male NOD/SCID mice. CRCs, Conditionally reprogrammed cells; NOD/SCID, Nonobese diabetic/severe combined immunodeficiency.

III. RESULTS

1. Establishment of CRCs from clinical specimens

We enrolled 46 patients who were pathologically diagnosed with PDAC. Among the 46 tumor samples, 40 samples were from patients who had undergone surgical resection. Four and two samples were from EUS-guided biopsy and percutaneous liver biopsy, respectively. The average success rate of establishing CRCs from PDAC tissues was approximately 34.8% (surgical resection, n = 13; EUS-guided biopsy, n = 2; and percutaneous liver biopsy, n = 1) (Table 2). The characteristics of the 16 patients are shown in Table 3.

Table 2. Success rates for CRC establishment of pancreatic cancer samples from either biopsy or surgical specimens

Sample type	Success, n	Failure, n	Success rate, %
EUS-biopsy	2	2	50.0%
Percutaneous biopsy	1	1	50.0%
Surgical resection	13	27	32.5%

CRCs, Conditionally reprogrammed cells; n, number; EUS, Endoscopic ultrasound

Table 3. Clinical characteristics of patients

Variables	Total (n=16)
Age at diagnosis, Mean ± SD	63 ± 9.3
Gender (%)	
Female	7 (43.7%)
Male	9 (56.3%)
T stage (%)	
T1,T2	0 (0%)
T3	13 (81.3%)
T4	3 (18.7%)
N stage (%)	
N0	3 (18.8%)
N1	10 (62.5%)
Differentiation (%)	
Moderate	12 (75.0%)
Poor	2 (12.5%)
Undifferentiated	2 (12.5%)
Resection margin (%)	
R0	10 (62.5%)
R1	3 (18.8%)
Adj. chemotherapy	
Gemcitabine	8 (50.0%)
FL	1 (6.3%)
Survival, day	
DFS (range)	126 (38-889)
OS (range)	387 (45-919)

Adj, Adjuvant; FL, 5-Fluorouracil and Leucovorin; DFS, Disease-free survival; OS, Overall survival; SD, Standard deviation

2. CRC characterization

Epithelial colonies were readily observed at 2 days and rapidly proliferated to reach confluence in approximately 7–14 days. Representative PDAC CRCs are shown in Figure 3. We characterized and confirmed the CRCs by the intense fluorescence of cytokeratin 19 and tumorigenesis *in vitro* (Figure 4 and 5). Immunofluorescence colony staining for CRCs was performed using an anti-cytokeratin 19 monoclonal antibody. The cytoplasm of cancer cells was clearly stained with this antibody. In the soft agar colony formation assay, CRCs formed colonies by day 16. Regarding key oncogenic mutations, KRAS mutation analysis was performed by PCR. Of the 7 cases analyzed, 5 showed KRAS mutations (Table 4).

3. In vivo tumorigenesis

In vivo, three CRCs (YPAC-2, 5, 16) were implanted in six NOD/SCID mice. All implanted CRCs showed tumor engraftment and grafted tumors were palpated in the bilateral flanks of 6 mice after 2–3 months. Grafted tumors were surgically removed. Hematoxylin and eosin staining showed that the implanted tumor tissues shared similar morphologies with the parent tumor tissue (Figure 7).

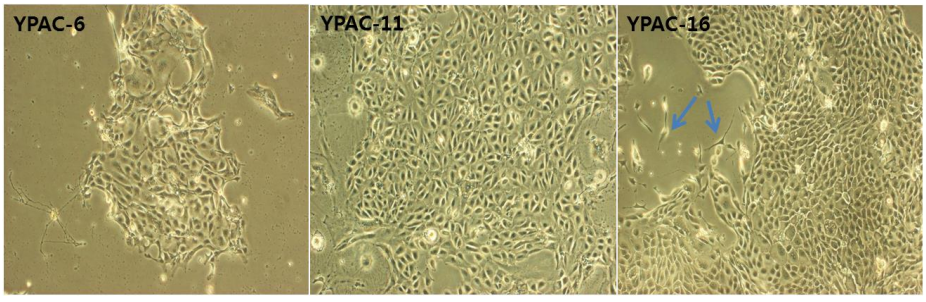

Figure 4. CRC formation after 14 days of culture. A–C representative colonies show various morphologies and arrow shows irradiated feeder cell. CRCs, Conditionally reprogrammed cells.

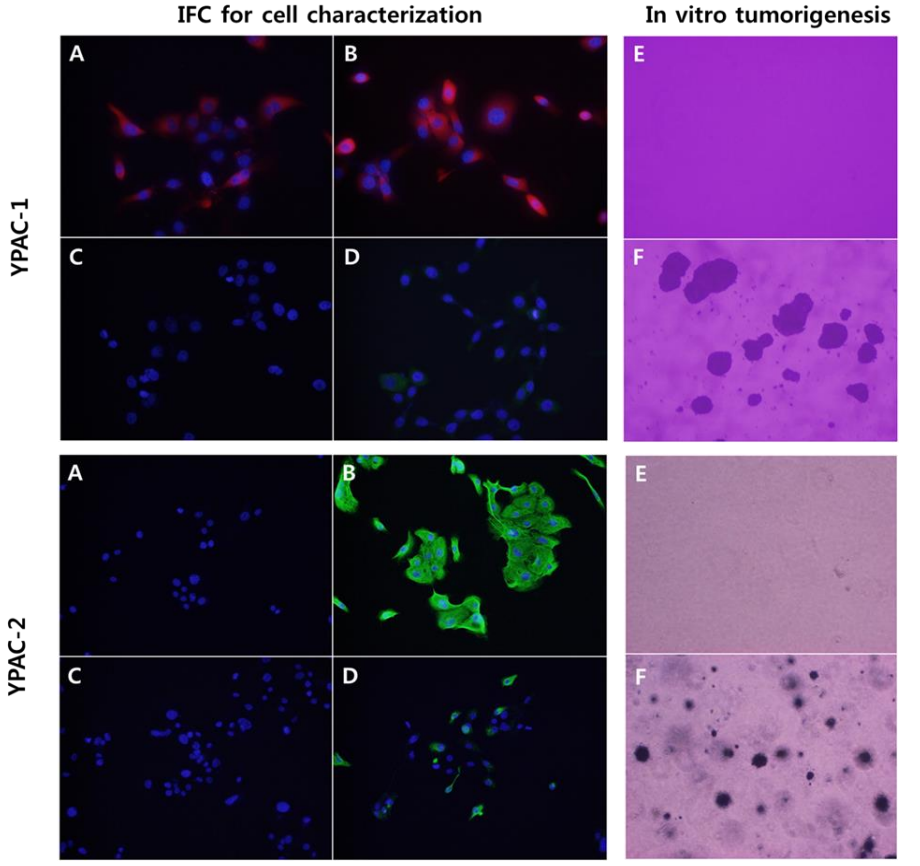

Figure 5. Characterization of the intensely fluorescent cells in CRCs. A–D) Fluorescent for cell characterization. (A) α-Amylase (sc-25562, rabbit polyclonal; Santa Cruz Biotechnology), dilution 1:100, resolution rate 200X; (B) cytokeratin 19 (A53-B/A2: sc-6278, mouse monoclonal; Santa Cruz Biotechnology), dilution 1:100, resolution rate 200X; (C) insulin (sc-8033, mouse monoclonal; Santa Cruz Biotechnology), dilution 1:100, resolution rate 200X; (D) vimentin (V9: sc-6260, mouse monoclonal; Santa Cruz Biotechnology), dilution 1:100, resolution rate 200X; (E,F) Soft agar colony formation assay. (E) Negative control, (F) tumor cell formation at day 16.

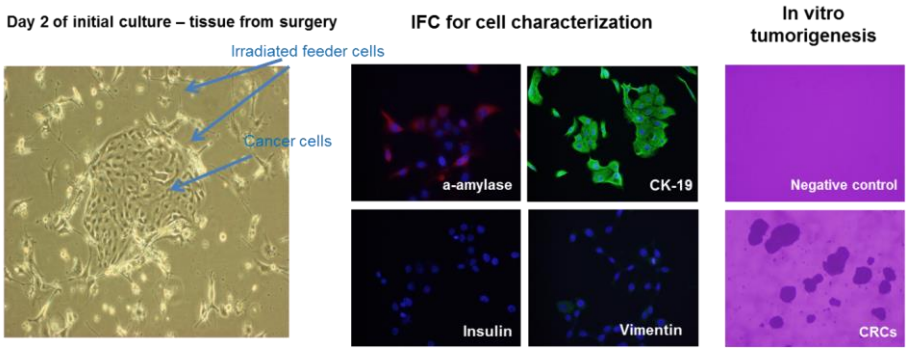

Figure 6. Representative data (YPAC-2). Percutaneous liver biopsy was performed in patients who were diagnosed with PDAC and showed liver metastasis and multidrug resistance. We characterized and confirmed CRCs by the intensely fluorescent cells in cytokeratin 19 and tumorigenesis *in vitro*. At day 2, cancer cell aggregates were noted. We confirmed *in vitro* tumorigenesis of CRCs. CRCs, Conditionally reprogrammed cells.

Table 4. KRAS mutation analysis by PCR

CRCs	KRAS	
	PCR	Exome sequencing
YPAC-1	-	-
YPAC-2	-	missense mutation, p.G12R
YPAC-3	-	-
YPAC-4	-	Wild
YPAC-5	missense mutation, p.G12D	missense mutation, p.G12D
YPAC-6	-	missense mutation, p.G12V
YPAC-7	-	missense mutation, p.G12D
YPAC-8	-	Wild
YPAC-9	Wild	Wild
YPAC-10	missense mutation, p.G12R	missense mutation, p.G12R
YPAC-11	-	Wild
YPAC-12	Wild	Wild
YPAC-13	missense mutation, p.G12V	missense mutation, p.G12V
YPAC-14	-	Wild
YPAC-15	missense mutation, p.G12V	missense mutation, p.G12V
YPAC-16	missense mutation, p.G12D	missense mutation, p.G12D

CRCs, Conditionally reprogrammed cells; PCR, polymerase chain reaction

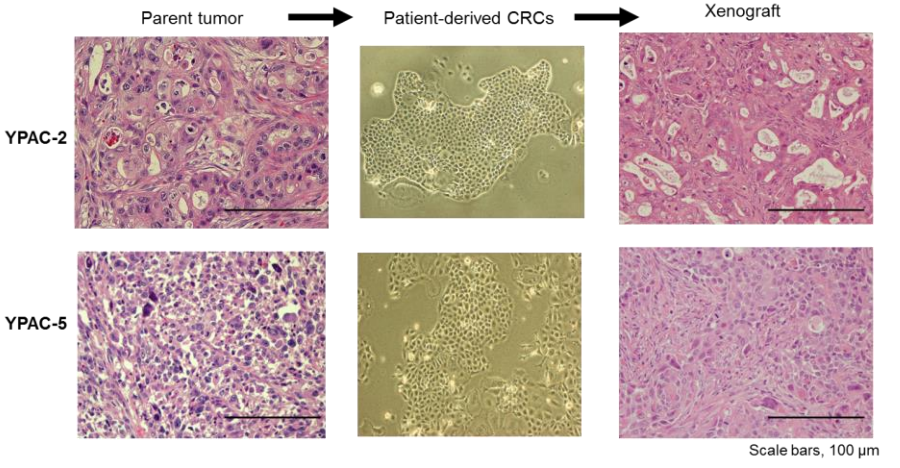

Figure 7. In vivo tumorigenesis and representative histology of xenografts.

Hematoxylin and eosin staining showed that implanted tumor tissue had similar morphology to parent tumor tissue.

4. Genetic characterization of patient-derived models

Targeted deep sequencing confirmed the preservation of primary tumor mutations in developed cell lines, which remained stable during extended passaging. We determined the relationship between the gene expression patterns observed in patient-derived cell lines and those observed in original PDAC specimens. Importantly, the genetic conservation of cell lines with PDX was presented as the overall maintenance of gene expression features. The preservation of somatic variants between tumor and PDX or cell lines was further evaluated by pairwise comparisons of the primary tumors with all models originating from the same primary tumor.

Before calling the somatic mutations, ContEst was performed to remove cross-contaminated samples from tumors and CRC samples. Therefore, we analyzed seven samples, except for the YPAC-7 sample with 20% estimated contamination. We confirmed that the genetic characteristics of cancer tissues were preserved in CRCs by comparing the AFs of somatic mutations found in cancer tissues and CRCs. In CRCs, the AFs of key oncogenic mutations present in both tumor and CRCs, including TP53, SMAD4, and KRAS, were found to be higher than or similar to in tumor (Figure 8). While *de novo* mutations are neglected and typically present at a low AF in the tumor, those in CRCs showed increased AFs (Figure 8B). This suggests that the original tumor characteristics can be amplified and expressed through the developed cell line.

The AF of TP53 and SMAD4 in the 4 CRCs samples was greater than 0.8,

except for the YPAC-5 sample, which showed a single copy loss during the process of tumor-driven CRC production. Similarly, multi-copy amplification of KRAS induced a homozygous mutation with a high AF (Table 5). Therefore, analysis of the cell lines indicated that they were genetically highly consistent with the tumors from which they were derived.

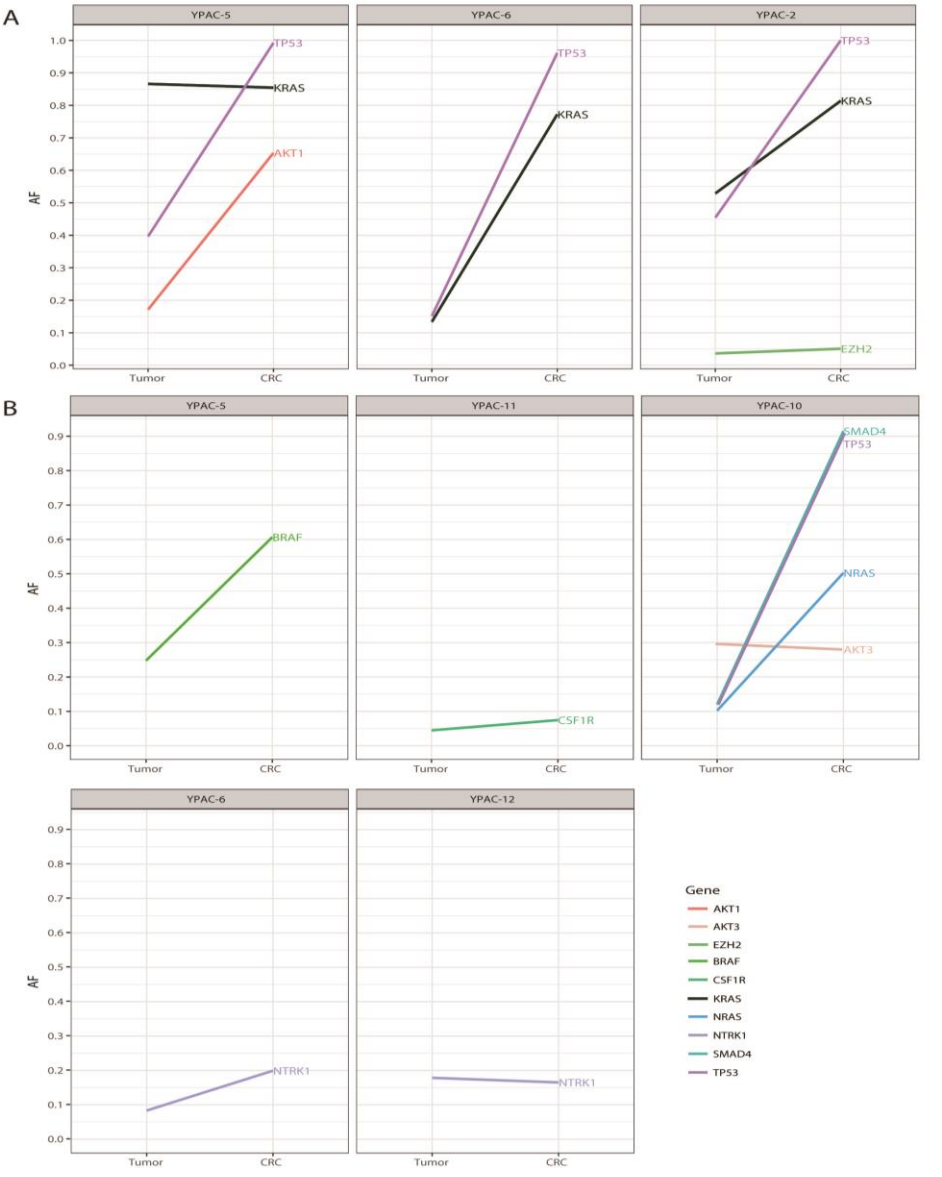

Figure 8. Genomic features between primary tumor and CRCs. (A) Allele frequency of key oncogenic mutations present in both tumor and CRCs. (B) Increased allele frequency between tumor and CRCs. AF, Allele frequency; CRCs, Conditionally reprogrammed cells.

Table 5. Copy number of high allele frequency mutations

Sample	Gene	Cancer			CRCs		
		log2	CN	depth	log2	CN	depth
YPAC-2	KRAS	0.91	4	68.447	0.88	4	567.485
YPAC-6	KRAS	0.10	2	52.517	0.74	4	66.487
YPAC-5	KRAS	1.98	8	543.244	1.51	6	1203.180
YPAC-10	SMAD4	-0.03	2	79.777	-0.75	1	125.935
YPAC-2	TP53	-0.21	2	327.378	-0.39	1	239.876
YPAC-6	TP53	-0.06	2	509.536	-0.46	1	162.260
YPAC-5	TP53	-0.10	2	581.210	0.14	2	547.204
YPAC-10	TP53	-0.08	2	425.817	-0.81	1	309.413

*CN, Copy number; CRCs, conditionally reprogrammed cells

5. Drug sensitivity assay

The growth of CRCs was analyzed in an MTT assay after treatment with various concentrations of gemcitabine for 0–72 h. The anti-proliferative effect of gemcitabine differed between patients according to their prognosis.

The YPAC-1, 9, 10, and 12 cell lines were treated with gemcitabine. As shown in Figure 9, gemcitabine dose-dependently decreased pancreatic cancer cell viability. The results of the cell viability indicated that the anti-proliferative effect of gemcitabine was significantly higher in YPAC-1 and 10 than in YPAC-9 and 12 (Figure 9). Progression-free survival (PFS) of YPAC-10 was more than 1 year, while that of YPAC-9 and 12 was less than 1 year. Furthermore, early liver metastasis occurred in YPAC-9 and 12. YPAC-1 showed poor prognosis with a PFS of less than 90 days and early liver and peritoneal carcinomatosis was noted. YPAC-1 showed a very large tumor size (>70 mm), and the patient was stage IV at diagnosis. Therefore, direct comparison with other patients is difficult because of other clinical confounding factors.

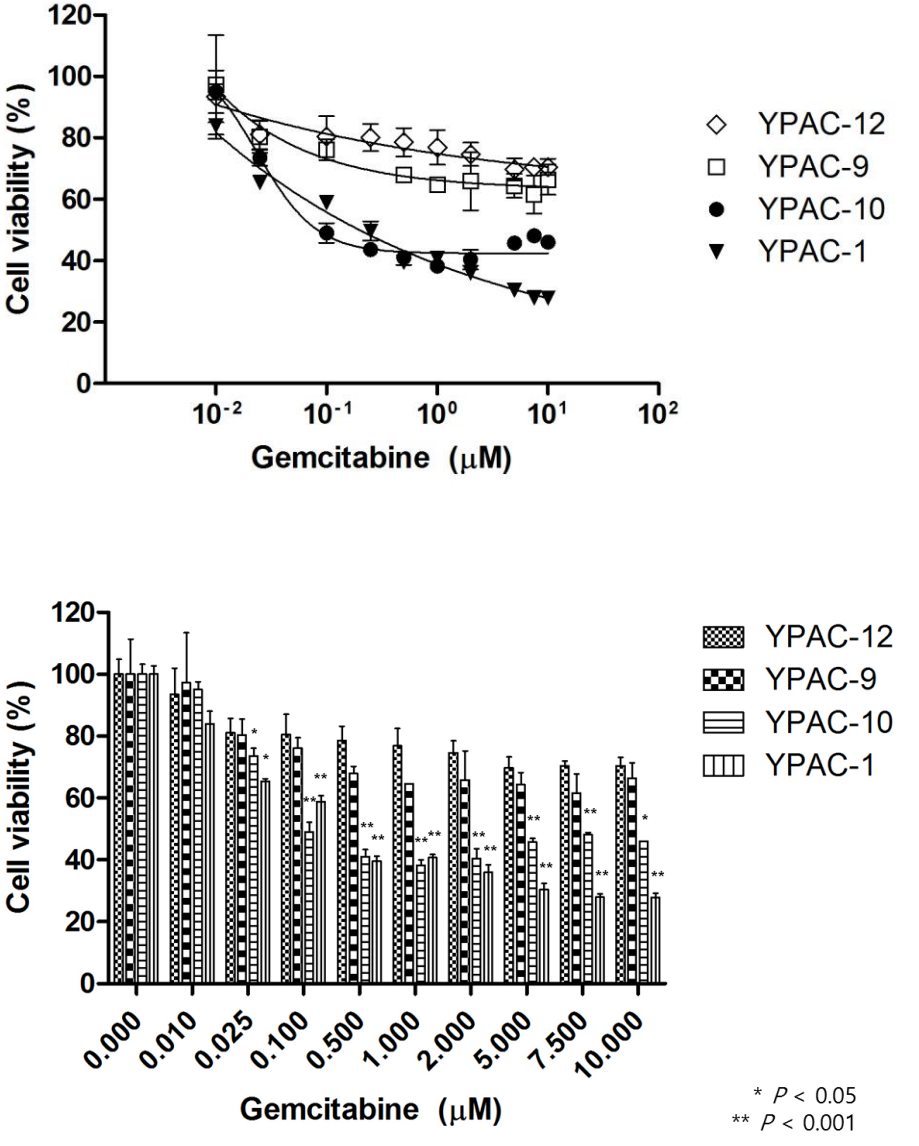

Figure 9. Cell viability assay and drug sensitivity. Effect of gemcitabine on human conditionally reprogrammed cells. The growth of pancreatic cells was analyzed via an MTT assay after treatment with various concentrations of gemcitabine over 0–72 h. The anti-proliferative effect of gemcitabine was greater in YPAC-1 and 10 than in YPAC-9 and 12.

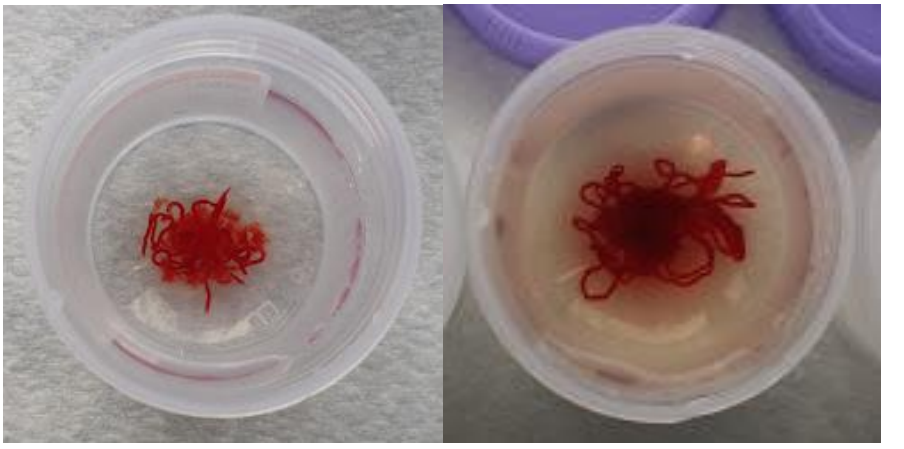

Figure 10. Tumor tissues from EUS-guided FNB. EUS-guided FNB was performed with a 20-gauge ProCore™ needle with a reverse bevel to obtain sufficient tumor samples. EUS-guided FNB, Endoscopic ultrasound-guided fine-needle biopsy.

IV. DISCUSSION

We established cell culture models derived from tumor samples of PDAC patients and then conducted genetic analyses of these cells. PDAC CRCs were developed with a 35% success rate from patient samples (biopsies and surgical specimens). The key oncogenic mutation present in the tumor tissue was reliably identified in the derived cell line.

Most patients present with inoperable advanced pancreatic cancer at initial diagnosis. At the time of pancreatic cancer diagnosis, only 15–20% of patients have a potentially resectable disease without evidence of major vessel involvement or extrapancreatic spread of the tumor.[2,8-11] In patients with inoperable advanced pancreatic cancer, a typical diagnostic tool for pancreatic cancer is EUS-guided fine-needle biopsy (FNB). EUS-guided FNB has the advantage of obtaining a histological specimen with better diagnostic performance. This approach can be used to obtain sufficient and qualified tumor samples for performing immunohistochemical analysis, accurate diagnosis, development of patient-specific cell lines, and genetic analysis. In this study, EUS-guided FNB was performed with a 20-gauge ProCore^{TM} needle with a reverse bevel to obtain sufficient tumor samples (Figure 10).[12-15]

In previous studies, the success rate of CRC culture was found to differ according to cancer type from 24.2 to 50%.[6,16-18] For pancreatic cancer, Liu et al. developed a total of 4 cell lines (2 cell lines, tumor) from 8 pancreas samples.[6] Natalya et al. reported a 100% (6/6) success rate for pancreatic cancer, but the

number of patients was too small to characterize the method.[17] The current study is the largest study to develop PDAC CRCs.

Histologic morphology was similar between matched samples and mutations of primary tumors and CRCs, with an increased AF observed in CRCs. KRAS, TP53, and SMAD4 are representative somatic mutations in pancreatic cancer.[19] In samples subjected to sequencing and PCR, these typical mutations were detected. Furthermore, key oncogenic mutations present at a low AF in the tumor showed an increased AF in CRCs. This may be a useful tool for detecting minor allele deficiency mutations in cancer.

Precision medicine focuses on delivering the most appropriate therapy to a patient through drug screening based on the clinical and molecular features of their disease. In the present study, CRCs showed the same characteristics as the original cancer samples. The drug sensitivity test showed that gemcitabine sensitivity was reliably associated with patient prognosis. A recent study evaluated the drug sensitivity to a large panel of clinical agents using conditionally reprogrammed patient-derived cancer cells and suggested a target drug.[17]

Recent genomic developments in PDAC treatment has provided various options for personalized treatment.[20-27] Studies divided patients into several groups according to their responsiveness to chemotherapy and survival.[21-24,28-30] Major limitations in implementing treatment strategies based on mutational status are the availability of the tumor for detailed sequence analysis in PDAC

research; (1) difficulty in primary cell culture owing to limited life span, (2) difficulty in tumor sequencing owing to the dominance of stromal cells, and (3) absence of an appropriate preclinical model preserving the molecular characteristics of tumor cells.

The CRC protocol has been demonstrated to easily establish patient-derived CRC cultures from lung cancer and prostate cancer tissues with the capacity to grow indefinitely without genetic manipulation. The technique uses irradiated mouse fibroblast cells and a Rho-associated kinase (ROCK) inhibitor (Y-27632) to propagate epithelial cells.[6,31-33] Compared to lung cancer[18] and prostate cancer,[16] previous studies using the CRC method are limited in pancreatic cancer. Recently, one study reported the establishment of PDAC cell lines using the CRC method.[17] However, in order to conduct genetic analysis, the establishment of genetically identical cell lines is necessary. In the present study, key mutations such as KRAS, TP53, and SMAD4 were preserved in CRCs.

The most commonly used method for immortalizing cells is transformation with SV40 virus large T antigen or overexpression of hTERT.[5,34] However, these genetic manipulations lead to genomic instability; after a few passages, there is an irreversible loss of critical biological and genetic characteristics compared with those of primary tissues from which they were derived.[35] Most primary cell cultures, regardless of the method used to generate them, are difficult to maintain, as they have a limited life span because of their gradual decrease in proliferation, eventually leading to senescence.

Regarding the differences between CRC method and other methods, the induction of CRCs is rapid (within 14 days) and results from reprogramming of the cell population rather than clonal selection, as is the case with conventional cell lines. Unlike embryonic stem cells and induced pluripotent stem (iPS) cells, CRCs from normal tissue do not express high levels of Sox2, Oct4, Nanog, or Klf4 and do not form teratomas in mice.[7] Moreover, CRCs maintain developmental potential and do not require intricate manipulation to differentiate into the tissue of origin. In tumor CRCs, phenotypic and genotypic features of the primary tumor are maintained; these CRCs have recently been used to identify an appropriate therapy for respiratory papillomatosis.[36] Therefore, the CRC method shows wide application potential and can be adapted for live bio-banking and basic research, as well as for diagnostic, therapeutic, and regenerative medicine. Both embryonic and iPS cells can be propagated *in vitro* and retain a normal karyotype. They can differentiate into several germ layers and show great potential in regenerative medicine. However, with limited exceptions, it is not feasible to precisely direct their differentiation into specific adult tissues. In addition, the use of human embryonic cells can, in some cases, be prohibited for ethical reasons. Generation of iPS cells from adult tissues typically requires transduction with exogenous genes that can alter cellular growth and differentiation properties, unlike the generation of CR cells, which maintain lineage commitment. More recently, a defined chemical method was used to generate iPS cells and, although promising, further testing and

verification are required before this reprogramming method can be used in regenerative medicine.

In the present study, this established system was used for direct comparison of the molecular and genetic profiles of pancreatic tumor tissue, enabling patient-specific treatment according to the subtype of PDAC. In addition, the capacity to culture metastatic lesions such as in the liver will enable identification of cellular changes that occur and potentially contribute to invasion and metastasis.

V. CONCLUSION

In conclusion, using the CRC method, we established PDAC cell cultures from patients. The use of matched patient-derived cells provided a unique *in vitro* model for studies of early PDAC. A critical finding of this study was the successful culture and representativeness of PDAC cells from surgical or biopsy specimens using a conditional reprogramming method. Sequencing analysis revealed that the CRCs maintained the mutations present in the original tumor cell. The PDAC CRC model is a novel *in vitro* model for testing chemosensitivity and genetic analysis of personalized treatment in patients with PDAC.

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ABSTRACT (IN KOREAN)

조건부 재편집 세포 배양법을 이용한 췌장암 환자 유래 종양 세포주의 형성 및 유전체 분석

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이 희 승

췌장암은 환자의 80-90%는 진단 당시에 이미 진행된 상태로 발견되며, 5년 생존율이 20% 미만으로 예후가 매우 나쁜 암이다. 최근 악성 종양에 대한 유전체 연구가 활발해짐에 따라 혈액암 및 대장암, 유방암 등의 대표적인 고형암 별로 보편적으로 동반되는 분자생물학적 이상 또는 표적에 대한 선택적인 억제가 가능한 표적치료제가 개발되고 있으며, 동일한 암종이라 하더라도 환자 개인별로 서로 상이한 유전자의 이상이 많고, 이러한 개인별 종양 관련 유전체 이상의 다양성으로 기존의 무차별적 항암화학요법보다 환자의 유전체 정보에 따른 선택적 맞춤형 치료가 가능해졌다. 하지만, 췌장암의 특성 상 종양 조직 획득의 어려움과 유전 변이의 다양성, 약물의 감수성

연구 및 내성 연구를 위한 실제 환자의 종양 세포의 분자생물학적 특성이 보존된 적절한 전임상모델의 부재로 인해 췌장암 환자의 치료에는 제약이 있었다.

최근 조지타운대학 Richard Schlagel 박사 연구팀이 극소량의 종양세포로부터 2주내 세포주를 수립할 수 있는 조건부 재편집 세포 배양법 (conditionally reprogrammed cell culture, CRC)을 개발하였으며, 이를 통해 실제 환자로부터 조직 생검을 통해 CRC를 단기간 내 배양하고, 새로운 치료 약제를 발굴하여 임상적으로 적용하여 그 효과를 입증하였다. 이를 통해 극소량의 조직 또는 종양 세포로부터, 빠른 시간 내 환자로부터 유래된 종양세포를 선택적으로 증폭 배양하여 세포주를 구축할 수 있는 시스템을 구축하였다.

이에 본 연구에서는 조건부 재편집 세포 배양법을 이용하여, 환자 유래 종양 세포주를 만들고, 유전체 분석을 통해 환자의 유전적 특성을 대변함을 확인하고자 하였다.

수술 조직 및 내시경 초음파 유도 조직검사를 통해 환자 검체를 확보하고 irradiated feeder cell과 Rho-associated kinase inhibitor을 함께 배양하여 세포주를 만들었다. Genetic level에서

검증을 위해 유전체 분석을 진행하였고, 면역력 저하 실험용 쥐에서 종양 형성을 확인하였다. 실험 결과 46명의 환자 중 16명 (34.8%)에서 조건부 재편집 세포 배양법을 활용한 환자 유래 종양 세포주를 확인하였고, 췌장암의 대표적인 유전 변이인 KRAS, TP53, SMAD4 변이를 확인하였다. 세포 실험과 동물실험 모두에서 종양 세포 형성을 확인하였고 조직학적으로 기존의 췌장암과 유사한 것을 확인하였다. 또한 약물 반응 스크리닝 검사를 통해 환자의 임상 예후와 유사하게 발생하는 세포주의 약제 반응성을 확인하였다.

본 연구를 통해 췌장암에서 전임상 모델의 하나로 조건부 재편집 세포 배양법을 이용한 환자 유래 세포주 형성을 성공적으로 확인하였고, 향후 췌장암 환자 맞춤형 치료를 위해 활용 가치가 크다.

핵심되는 말: 조건부 재편집 세포 배양법, 췌장암, 차세대 염기 서열 분석, 환자 유래 종양 세포