The Development of Cancer Stem Cell Specific Aptamers for Pancreatic Cancer

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The Development of Cancer Stem Cell Specific Aptamers for Pancreatic Cancer

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김윤진

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CSC	Cancer Stem Cell
CTC	Circulating Tumor Cell
hMSC	human Mesenchymal Stem Cell
SELEX	Systematic Evolution of Ligands Exponential Enrichment

ABSTRACT

The Development of Cancer Stem Cell Specific Aptamers for Pancreatic Cancer

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(Directed by Professor Si Young Song)

Pancreatic cancer remains one of the most common and lethal cancer. Most patients (80%) present with inoperable advanced pancreatic cancer at initial diagnosis and their early diagnosis is a significant unmet challenge. Recent studies indicate that cancer, including pancreatic cancer, is initiated and propagated by cancer stem cells (CSCs). CSCs are responsible not only for the pathogenesis of cancer but also for the heterogeneity, malignant degree, anti-cancer therapy resistance, and recurrence of tumors. Therefore, identification of CSCs using convincing biomarkers may be a crucial stepping stone for overcoming this disastrous pancreatic cancer.

Here, we investigated pancreatic CSC-associated aptamers as a novel tool for diagnosis and therapeutic agents.

Aptamers that bind to pancreatic cancer stem cell were developed by modified cell-SELEX method. Positive selection was performed by the sphere cells generated by pancreatic cancer cell line, HPAC, and then the aptamer pool was negatively selected by

pancreatic normal cell line, HPDE.

After cell-SELEX, the aptamer 1 and 146 showing high specificity upon the K_D values with 22.18 nM and 22.62 nM were selected. These two aptamers were validated by binding to HPAC sphere cells but to HPDE cells and both aptamers showed specificity to HPAC sphere cells only.

Aptamer-positive cells showed high expression levels of CSC-associated genes compared to the aptamer-negative cells by FACS analysis. Co-localization of CD44, CD24, ESA and CD133 was also observed in the aptamer-positive cells by confocal microscopy.

In the present study, we determined that these two pancreatic CSC-specific aptamers may be potential candidates for novel diagnostic markers, CSC-targeting drug delivery, or circulating-tumor-cell detection.

Key words: pancreatic cancer, cancer stem cell, aptamer

The Development of Cancer Stem Cell Specific Aptamers for Pancreatic Cancer

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I. INTRODUCTION

The prognosis for pancreatic cancer patients is extremely poor. Patients with locally advanced disease have a median survival time of 8 to 12 months, and patients with distant metastasis have significantly worse outcomes, with a median survival time of only 3 to 6 mo. Furthermore, most patients present with inoperable advanced pancreatic cancer at initial diagnosis.[1]

On the other hand, the identification of early diagnostic or predictive biomarkers remains a major challenge in pancreatic cancer. The most clinically useful biomarker is carbohydrate antigen CA 19-9. CA 19-9 can be used in diagnosis, screening, staging, and as a prognostic marker after resection.[2] However, CA19-9 is not tumor-specific marker and its low positive predictive value makes it a poor biomarker for screening.[3]

Recent studies showed that pancreatic cancer is initiated and

propagated by CSCs. CSCs may be derived from normal stem cells, progenitor cells, or differentiated cells and have both cancerous and stem cell-like characters.[4] CSCs present in only small number of tumor mass, however, CSCs can initiate tumorigenesis and also associated with self-renewal, drug resistance, and metastasis.[5] The conventional anticancer therapies are usually targeting to both differentiated and differentiating cells and not to CSCs. If the biomarkers specific to CSCs can be elucidated, it will be very helpful for the early diagnosis, overcome chemoresistance, or response and recurrence expectation.

The needs for the comprehensive approach targeting cancer together with CSCs may introduce the better outcome in early diagnosis and cancer therapy. Due to the importance of targeting CSCs there have been many studies on identifying specific CSCs surface markers. However, a solid marker has not been decided as a CSC marker in pancreatic cancer. To overcome this situation, we decided to develop the pancreatic CSC-associated aptamers. Aptamer is a single strand of either an oligonucleic acid or a peptide molecule which doesn't have a molecular function but recognize and bind to a specific target structure. Aptamers targeting CSC-specific surface markers such as ErbB2[6] or EpCAM[7] have been generated but neither are the absolute CSC markers, we designed aptamers specific to the CSC-associated cell surface using Cell-SELEX method[8] and evaluated its role as a CSC-associated targeting biomarkers.

II. MATERIALS AND METHODS

1. Cell culture and reagents

HPAC cell line (CRL2119, human pancreatic adenocarcinoma) was purchased from the American Type Culture Collection (ATCC, Manassas, Virginia, USA) and was cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, Invitrogen, Waltham, Messachusetts, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics (Invitrogen). MiaPaCa-2 cell line (human pancreatic adenocarcinoma) was obtained from ATCC and was cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% FBS, 2.5% horse serum, and antibiotics. HPDE cell line was kindly donated by Dr Ming Sound Tsao and was cultured in keratinocyte medium (invitrogen). Human bone marrow derived mesenchymal stem cell line (hMSC) was obtained from Cell Therapy Center (Severance Hospital, Republic of Korea) and was cultured in DMEM medium supplemented with 10% FBS and antibiotics. Cells were trypsinized and resuspended at a concentration of 1500 cells/mL in DMEM/F12 media supplemented with epidermal growth factor (10 ng/mL; R&D Systems Inc, Minneapolis, Minnesota, USA), fibroblast growth factor (10 ng/mL; R&D Systems Inc), insulin transferring selenium (Invitrogen) and 0.5% FBS. Cell suspensions were seeded in T75 ultra-low attachment plates at 37 ℃ for 7 days and were then collected. The aptamer washing buffer contained 4.5 g/L glucose and 5 mM $MgCl_2$, in Phosphate-buffered saline (PBS). Binding buffer used for selection was prepared by adding yeast tRNA (0.1

㎎/mL; sigma-aldrich, St Louis, Missouri, USA) and Bovine Serum Albumine (BSA; 1 ㎎/mL; Invitrogen) in to the wash buffer to reduce background binding.[8] The Taq polymerase and dNTPs used in Polymerase Chain Reaction (PCR) were obtained from Takara (Shiga, Japan).

2. SELEX Primers and Library

A FAM-labeled forward primer (5'-AAGGAGCAGCGTGGAGGATA-3') and a poly-dA tagged reverse primer (5'-(A)20-C18-ACCACGACGACACACCCTAA-3') were used in the asymmetric PCR.[8, 9] The SELEX library consisted of a central randomized sequence of 45 nucleotides (nt) flanked by two 20 nt primer hybridization sites (5'-AAGGAGCAGCGTGGAGGATA-N45-TTAGGGTGTGTCGTCGTGGT-3'). After incubation, the double-stranded PCR product was denatured by heating and was separated single-stranded DNA (ssDNA) in polyacrylamide gel to separate the FAM-conjugated forward ssDNA strand from the poly-dA reverse ssDNA strand, after which the sense ssDNA strand could be used for next round selection.

3. Cell-SELEX protocol

The process of cell selection was as follows: ssDNA pool (200 ㎰ol) dissolved in 400 μL of binding buffer was denatured by heating at 95 ℃ for 5 min and cooled on ice for 10 min before binding. HPAC derived sphere cells were dissociated into single

cell for incubation. Then the ssDNA pool was incubated with 1*10^6 dissociated sphere cells on ice and shaken at 200 rpm for 45 min. After washing, the bound DNAs were eluted by heating at 95 ℃ for 5 min in 300 μL of binding buffer. After centrifugation, the supernatant was desalted and then amplified by PCR (20 cycles of 60s at 95 ℃ for denaturing, 30s at 67 ℃ for annealing, and 60s extension at 72 ℃, followed by 5 min extension at 72 ℃). The selected forward FAM-labeled ssDNA was separated from the poly-dA reverse ssDNA strand by polyacrylamide gel. To evolve the aptamers with high affinity and specificity, the wash strength was enhanced gradually by extending the wash time (up to 10 min) and increasing the volume of wash buffer (up to 5 mL) and the number of washes (up to 4 times). After 16 rounds of selection, the enriched ssDNA pool was PCR amplified using unmodified primers and cloned into Escherichia coli using the TA cloning kit (invitrogen). The aptamer candidate sequences were obtained from the Macrogen (Seoul, Korea) (Figure 1).

aptamer	Variable N45 sequences from 21th to 65th position (5' – 3') in the ssDNA aptamers																																												
position	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65
1	T	C	C	G	T	T	C	G	T	A	C	T	C	G	C	A	A	G	C	T	G	A	T	C	C	G	C	G	G	C	G	C	G	A	C	T	T	G	T	G	A	C	A	A	
154	T	C	C	G	T	T	C	G	T	A	C	T	C	G	C	A	A	G	C	T	G	A	T	C	C	G	C	G	G	C	G	C	G	A	C	A	T	G	T	G	A	C	A	A	
163	T	C	C	G	T	T	C	G	T	A	C	T	C	G	C	A	A	G	C	T	G	A	T	C	C	G	C	A	G	C	G	C	G	A	C	T	T	G	T	G	A	C	A	A	
27	T	C	C	G	T	T	C	G	T	A	C	T	C	C	C	A	A	G	C	T	G	A	T	C	C	G	C	G	G	C	G	C	G	A	C	T	T	G	T	G	A	C	A	A	
39	T	C	C	G	T	T	C	G	T	A	C	T	C	G	C	A	A	G	C	T	G	A	T	C	T	G	C	G	G	C	G	C	G	A	C	T	T	G	T	G	A	C	A	A	
23	T	C	C	A	T	T	A	G	T	C	T	A	A	A	A	C	A	C	A	G	C	C	T	T	A	G	A	G	C	C	G	G	G	A	C	C	G	G	A	G	C	C	G	C	
23F	T	C	C	A	T	T	C	G	T	G	T	A	A	A	A	G	A	C	A	G	C	A	G	T	A	G	A	G	C	C	G	G	G	A	C	C	G	G	T	G	C	C	A	A	
146	A	C	C	A	C	T	A	G	T	C	T	A	A	A	A	G	A	C	A	G	C	C	G	T	A	G	A	G	C	C	G	G	G	A	C	C	G	G	T	G	C	C	A	C	
19	A	C	C	A	C	T	A	G	T	C	T	A	A	A	A	G	A	C	A	G	C	C	G	T	A	G	A	G	C	C	G	G	G	A	C	C	G	G	T	G	C	C	G	C	
25	A	C	C	A	T	T	A	G	T	T	T	A	A	A	A	G	A	C	A	G	C	C	G	T	A	G	A	G	C	C	G	G	G	A	C	C	G	G	T	G	C	C	A	C	
25 F	A	C	C	A	T	T	A	G	T	T	T	A	A	A	A	G	A	C	A	G	C	C	G	T	A	G	A	G	C	C	G	G	G	A	C	C	G	G	T	G	C	C	A	A	
28	A	C	C	A	T	T	A	G	T	C	T	A	A	A	A	G	A	C	A	G	C	C	G	T	A	G	A	G	C	C	G	G	G	A	C	C	G	G	T	G	C	C	A	C	
132F	A	C	C	A	T	T	A	G	T	G	T	A	A	A	A	G	A	C	A	G	C	C	G	T	A	G	A	G	C	C	G	G	G	A	C	C	G	G	T	G	C	C	A	C	
171	A	C	C	A	C	T	A	G	T	C	T	A	A	A	A	G	A	C	A	G	C	C	G	T	A	G	A	G	C	C	G	G	T	A	C	C	G	G	T	G	C	C	A	C	
40	G	C	G	C	T	C	C	C	A	A	C	G	G	C	C	A	C	A	T	A	C	C	A	G	T	A	C	A	C	G	C	G	G	G	A	C	C	G	C	G	G	C	C	T	G
7	G	C	G	C	G	A	C	G	C	A	A	A	A	A	G	G	G	A	T	G	T	G	A	A	G	T	C	A	G	G	C	T	A	G	T	A	G	G	G	C	A	T	C	G	
106	T	G	G	C	C	T	C	G	G	T	C	C	C	G	C	A	A	G	T	A	G	A	G	G	C	A	T	G	T	C	G	C	C	G	T	T	G	G	G	A	G	C	A	A	
140	T	G	G	T	C	A	C	C	T	A	C	A	A	C	A	T	T	A	C	A	G	G	T	C	A	G	G	C	T	C	A	T	T	A	A	G	C	G	T	T	G	A	A		
108	G	G	G	G	A	T	A	G	C	G	G	G	T	G	T	A	T	A	T	T	T	A	A	G	C	T	T	T	A	C	G	G	C	T	G	T	G	T	G	G	T	C	T	G	
113	G	G	G	C	T	C	C	C	A	A	C	G	G	T	C	A	C	A	T	A	C	C	A	G	T	A	C	A	C	G	C	G	G	A	A	C	C	G	C	G	G	C	C	T	G
127	C	G	C	G	A	G	A	A	C	T	G	T	G	C	A	A	C	T	A	C	G	G	A	C	G	G	T	A	C	T	A	G	C	T	T	G	T	G	G	G	G	T	G	C	T
15	C	C	G	A	T	G	C	C	A	G	A	C	A	C	A	A	T	T	G	T	T	A	T	C	C	C	G	C	G	C	A	T	G	C	C	A	C	C	A	T	C	C	G	T	C

Figure 1. Conserved sequences of aptamer. After the cell-SELEX process, 94 clones were sequenced and we found 22

individual sequences. Sequences shown in the boxes are the conserved sequences matched to their positions; the consensus sequences are highlighted in yellow, pink, sky-blue, blue, and purple. The sequences were similar in the secondary structure for each group. Among these 94 clones, yellow was the most frequently expressed group.

4. Flow cytometry

To monitor the enrichment of aptamer candidates during selection process, FAM-labeled ssDNA pool was incubated with $1*10^5$ dissociated sphere cells in 200 μL of binding buffer containing 10% FBS on ice for 30 min. Cells were washed twice with 0.7 mL of washing buffer and suspended in 0.4 mL of binding buffer. The fluorescence was determined with a LSR Ⅱ flow cytometer (Becton Dickinson immunocytometry systems, San Jose, California, USA) by counting 10,000 events. The initial dissociated sphere cells were used as background sample. The binding affinity of selected aptamers was measured by incubating HPAC derived dissociated sphere cells, HPDE cells ($1*10^5$) or hMSC cells with a series of FAM-labeled aptamers dissolved in a 200 μL volume of binding buffer containing 10% FBS on ice for 30min. Cells were then washed twice with 0.6 mL washing buffer, after which the cells were suspended in 0.4 mL binding buffer and subjected to flow cytometric analysis. The aptamer binding cells were counted and used to calculate the equilibrium dissociation constants (K_D) of

the aptamer-cell interaction. By using the prism software, the apparent K_Ds of the aptamer-cell interaction were evaluated according to the dependence of fluorescence intensity of specific binding on the concentration of the aptamers, which was well-known as the one binding site equation $Y=BmasX/(K_D+X)$, where the Y represents the bound fraction, the Bmax is the saturated binding, and the X is the concentration of ligand.

5. Immunofluorescence

A total of $1*10^6$ parental HPAC cells were incubated with 200 nM FAM-labeled random sequence, aptamer 1 or 146 and 1 μL of hoechst in 200 μL of binding buffer containing 10% FBS on ice for 30 min. Cells were washed twice with 500 μL of binding buffer and suspended in 200 μL of binding buffer. Cell suspension of 20 μL was dropped on a thin glass slide that was placed above the 60X objective of a confocal microscope, and then covered with a cover slip. Cell imaging was performed on an Olympus confocal microscope (LSM700, Carl Zeiss, Oberkochen, Germany).

To confirm the relationship between aptamer-positive cells and pancreatic CSC markers, 200 nM of FAM-labeled aptamer 1 or 146, as well as 10 μL of PE-labeled anti-CD44, CD24, EpCAM, or CD133 (BD biosciences) were used.

To identify circulating tumor cells (CTCs), cells were fixed with 4% paraformaldehyde (PFA) for 20 min, and washed with PBS three times for 10 min. The cells were subsequently permeabilized with 0.2% Triton X-100 (sigma-aldrich) in PBS, washed again three

times for 10 min, and stained with PE-labeled anti-CD133, cy5-aptamer 1, and FITC-cytokeratin (BD bioscience) for the identification process.

6. Isolation of CTCs (OncoQuick)

Blood samples from metastatic pancreatic cancer patients were used for CTC analysis. Five patients with metastatic pancreatic cancer were enrolled according to a separate protocol approved by the Institutional Review Board. Human blood samples were collected in EDTA tubes. Blood samples were processed within 6 hr after collection using OncoQuick columns as per manufacturer's recommendations, using 10 mL blood with 5 mL buffer (PBS + 0.5% BSA). Columns were centrifuged at 1600×g for 20 min at 4 ℃ (with slow acceleration and no break). The liquid above the porous barrier was carefully removed and transferred to a 50 mL conical centrifuge tube, and inner OncoQuick tube wall and porous barrier were washed three times with 5 mL was buffer, which was then withdrawn and transferred to the same conical centrifuged at 200×g for 10min at 4 ℃. About 45 mL of the supernate was withdrawn and discarded, and 45 mL of wash buffer was added, mixed, and the tube was recentrifuged as before. For immunofluorescence assay, the pellet was resuspended in binding buffer.[11]

7. Quantitative RT-PCR

For sampling, 200 μM of FAM-labeled aptamer 1 was incubated

with parental HPAC cells in binding buffer containing 10% FBS on ice for 30 min. Cells were washed twice with washing buffer and then resuspended in binding buffer. Fluorescence was determined with an Aria Ⅱ flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). After either the aptamer 1 or 146 positive and negative cells were sorted out, samples were prepared by Cells-to-CT Kit (Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA).

Seven target genes were analyzed: CD133, CD24, ALDH1, nanog, indian hedgehog (IHH), e-cadherin, n-cadherin, and U6 (used as a control).

Primers used for qRT-PCR were as follows: the CD133 forward primer was 5'-GCTCAGACTGGTAAATCCCC-3' and the reverse primer was 5'-GACTCGTTGCTGGTGAATTG-3'.The CD24 forward primer was 5'-ACCACGAAGAGACTGGCTGT-3' and the reverse primer was 5'-CCCACGCAGATTTATTCCAG-3'. The ALDH1 forward primer was 5'-AGCAGGAGTGTTTACCAAAGA-3' and the reverse primer was 5'-CCCAGTTCTCTTCCATTTCCAG-3'. The E-cadherin forward primer was 5'-TGCCCAGAAAATGAAAAAGG-3' and the reverse primer was 5'-GTGTATGTGGCAATGCGTTC-3'. The N-cadher in forward primer was 5'-ACAGTGGCCACCTACAAAGG-3' and the reverse primer was 5'-CCGAGATGGGGTTGATAATG-3'. The U6 forward primer was 5'-AACGCTTCACGAATTTGCGT-3' and the reverse primer was 5'-CTCGCTTCGGCAGCACA-3'. The primers for nanog and IHH genes were purchased from Bioneer.

The amplification was carried out in a total volume of 10 μL containing DNA Master SYBR green I (Roche Molecular Systems, Basel, Switzerland) and 2 μL of 1:10 diluted cDNA. PCR reactions were prepared in triplicate and heated to 95 °C for 10min followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 65 °C for 5 s, and extension at 72 °C for 20 s. All PCR reactions were performed in duplicate. The relative factor of differential expression was the ratio of the normalized value of each aptamer-positive sample or target gene to the normalized value of each aptamer-negative sample.

III. RESULTS

1. Development of aptamers using Cell-SELEX method

To generate aptamers with high affinity and specificity to the CSCs, Cell-SELEX method was used. The Cell-SELEX is using random sequence DNA library binding with dissociated sphere cells as positive selection, and then elutes the target bound DNA strands. Eluted DNA was amplified by PCR reaction and double stranded was denatured to a single stranded. By applying the same processes for 16 times and when the approximately 90% of DNA bound to the target, the overall process was ended and the eluted DNA was obtained (Figure 2A).

Among the 16 rounds, 13 rounds were the positive selection with dissociated sphere cells, and 3 further rounds were the negative selections. The negative selection includes the binding with pancreatic normal cell lines, and FACS sorted CD24-/CD44-/EpCAM- triple marker negative cells.

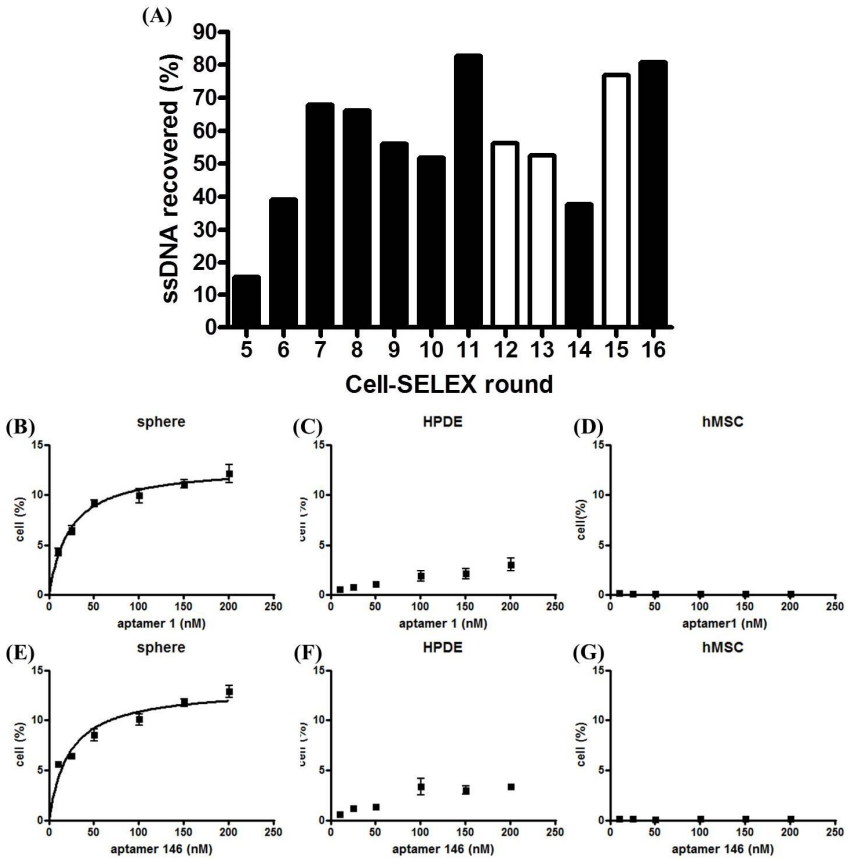

Figure 2. Cell-SELEX process and affinity test. (A) Single-stranded DNA aptamers that bind to pancreatic CSCs were developed by a modified Cell-SELEX method. Positive selection (black bar) was performed by sphere cells generated by the HPAC cell line. Negative selection (empty bar) was performed by $CD44^-/CD24^-/EpCAM^-$ HPAC cells or a pancreatic normal cell line, HPDE.

(B-G) Sensitivity and specificity tests. Two aptamers, aptamer 1 and aptamer 146, were found to be highly sensitive to pancreatic CSCs. (B, E). Also, these two aptamer variants showed higher specificity to sphere cells followed by HPDE (C, F) and hMSC(D, G).

2. Identification of pancreatic CSC-associated aptamers

After the Cell-SELEX process, we cloned 22 aptamers (Figure 1). Among 22 aptamer, we sorted 7 sequences (Table 1) that with no sequence similarities, and performed affinity test using flow cytometry assay (Table 2, Figure 2B and E). In our results, 7 aptamers were sensitive based on their affinity to dissociated sphere cells with equilibrium dissociation constant (K_D) values ranging from 22.18 nM to 65.79 nM using the Prism software. Finally, aptamer 1 and aptamer 146 were selected, since they are highly sensitive to stemness-enriched cancer cells in pancreatic cancer. In addition, we confirmed whether aptamer 1 and aptamer 146 were specific to dissociated-sphere cells, in comparison to HPDE cells and hMSC. (Figure 2C, D, F and G). The Figure 3 (A and B) show the predicted secondary structure of aptamer 1 and aptamer 146 done by m-fold program.

Table 1. Individual sequences of 7 aptamers

Aptamer	Sequence
1	AAGGAGCAGCGTGGAGGATATCCGTTCGTACTCG CAAGCTGATCCGCGGCGCGACTTGTGACAATTAG GGTGTGTCGTCGTGGT
146	AAGGAGCAGCGTGGAGGATAACCACTAGTCTAA AAGACAGCCGTAGAGCCGGGACCGGTGCCACTTA GGGTGTGTCGTCGTGGT
25	AAGGAGCAGCGTGGAGGATAACCATTAGTTTAAA AGACAGCCGTAGAGCCGGGACCGGTGCCACTTAG GGTGTGTCGTCGTGGT
40	AAGGAGCAGCGTGGAGGATAGCGCTCCCAACGG CCACATACCAGTACACGCGGGACCGCGGCCTGTT AGGGTGTGTCGTCGTGGT
23	AAGGAGCAGCGTGGAGGATATCCATTAGTCTAAA ACACAGCCTTAGAGCCGGGACCGGAGCCGCTTAG GGTGTGTCTGCTTGGT
15	AAGGAGCAGCGTGGAGGATACCGATGCCAGACA CAATTGTTATCCCGCGCATGCCACCATCCGTCTTA GGGTGTGTCGTCGTGGT
108	AAGGAGCAGCGTGGAGGATAGGGGATAGCGGGT GTATATTTAAGCTTTACGGCTGTGTGGTCTGTTAG GGTGTGTCGTCGTGGT

Among 22 selected aptamers, we sorted 7 sequences with no sequence similarities or predicted secondary structure similarities.

Table 2. Affinity measurements of 7 aptamers

Aptamer	K_D
1	22.62±8.25
146	22.18±9.6
25	65.79±23.83
40	54.28±36.47
23	49.69±24.98
15	45.18±13.39
108	62.8±31.84

The seven aptamers showed high affinities to sphere cells with K_D values from 22.18 nM to 65.79 nM.

3. Visualizing pancreatic cancer stem cells by FAM-labeled aptamer 1 or aptamer 146

To visualize the binding of the aptamers to the cells, we performed immunofluorescence assay by binding the FAM-labeled aptamer 1, aptamer 146 and random sequences to parental HPAC cell line and visualized under the fluorescence microscope. Aptamers were successfully bound the paraental HPAC cell surface, however, the random sequence as a negative control didn't bind to the cell surface (Figure 3C, D).

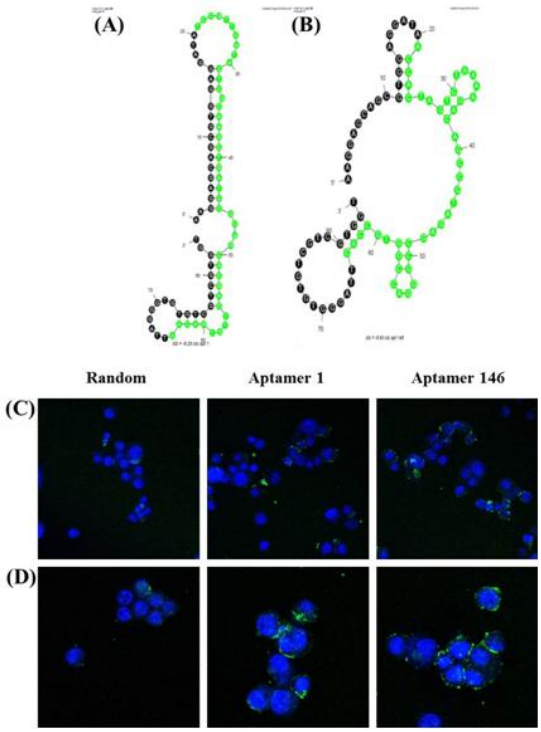

Figure 3-A and B. Predicted secondary structures. Secondary structures of aptamer 1 and 146 were predicted by the m-fold program. Aptamers were composed of a total of 84 basepairs with a central variable region of 44 base pairs (green circles) flanked by primer binding regions of 20 base pairs each (black circles). (A) apatmer 1 (B) aptamer 146

Figure 3-C and D. Fluorescence confocal images of cells. HPAC cells were incubated with FITC-labeled aptamer 1 and 146. FITC-labeled random sequence, which does not bind to HPAC cells, was used as a control (C: 20x, D: 40x).

4. Differentially expressed genes between aptamer-positive and -negative cells

We then sorted out the cells that the aptamers bound to (aptamer-positive) from those that did not bind to the aptamers (aptamer-negative) among parental HPAC and MiaPaCa-2 cell lines using FACS analysis and compared the gene expression between these two groups (Figure 4).

Aptamer 1-positive cells in the parental HPAC cell line were found to have increased levels of CSC-related genes, such as CD133, CD24, and ALDH1 (Figure 4A). Similarly, aptamer1-positive cells in the MiaPaCa-2 cell line were also found to have increased levels of CD133, CD24, IHH, and nanog genes (Figure 4B). Aptamer 146-positive cells were found to have increased levels of CSC-associated genes such as CD133, CD24, IHH, and nanog genes in both HPAC and MiaPaCa-2 cell lines (Figure 4C and D). Additionally, the aptamer 1-positive cells were found to have more n-cadherin than e-cadherin genes. To summarize, the qRT-PCR results indicated that levels of CD133 and CD24 genes, known as CSC markers, were both increased, while the levels of EMT-related genes such as n-cadherin and e-cadherin were changed only in aptamer 1-positive cells.

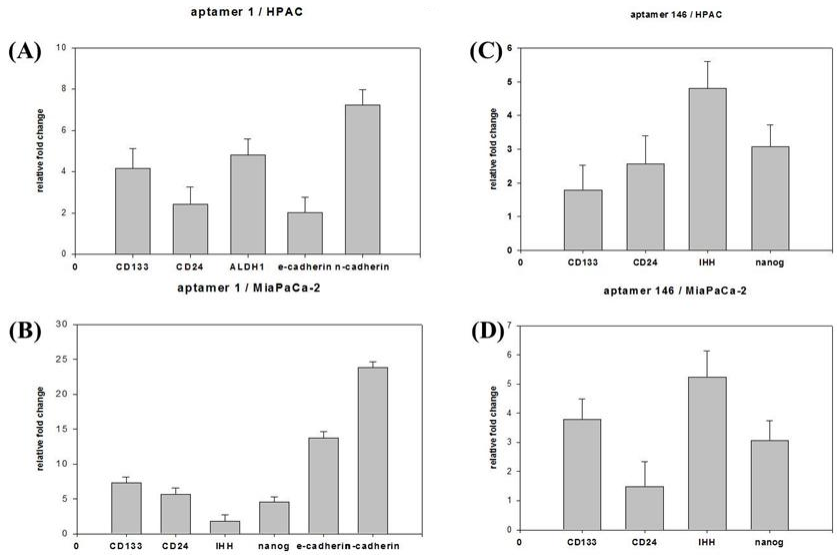

Figure 4. Differentially expressed genes between aptamer-positive and -negative cells were tested by qRT-PCR analyses. The qRT-PCR analyses revealed that aptamer 1- and 146-positive cells had higher expressions of CSC-related genes than aptamer-negative cells. The internal control was U6, and every gene expression of aptamer-positive cells was normalized using expressions of aptamer-negative cells. (A: aptamer 1 in HPAC cell line, B: aptamer 1 in MiaPaCa-2 cell line, C: aptamer 146 in HPAC cell line, D: aptamer 146 in MiaPaCa-2 cell line, $p < 0.05$)

5. Relationship between aptamer-positive cells and pancreatic CSC markers

This result was further confirmed by IF assay by confocal microscope (Figure 5). We bound both CD24 and aptamer 1 or 146 to parental HPAC cells, and after merging the data, we observed that CD24-positive cells and highly expressed cells had also bound to the aptamers. FAM-labeled aptamer 1 or aptamer 146 with PE-conjugated CD24 antibody and Hoechst dye was used to visualize the cell nucleus (Figure 5A).

We bind HPAC cells with CD44 and found out that most of the HPAC cells are expressing CD44 on cell surface. However, the cells that are highly expressing CD44 was observed and when we merge only the cells with highly expressing were bind with the aptamer 1 or 146 (Figure 5B).

Binding HPAC cells with EpCAM, large number of the HPAC cells are expressing EpCAM on cell surface, and aptamer 1 or 146 binds with the cells that are highly expressing EpCAM but not all to it. In other words, among the cells expressing high EpCAMs, aptamers 1 or 146 selectively binds to it (Figure 5C). The correlation between CSC marker expression (CD24+/CD44+/EpCAM+) and aptamer binding to the cells was quantitatively evaluated by flow cytometry (Figure 6).

CD133 is also known as a CSC candidate marker and in our previous FACS data tells that the expression of CD133 in HPAC is rare. When we bind with CD133 with aptamer 1 or 146, we can clearly see the co-localization of the expression from the merged

data (Figure 5D).

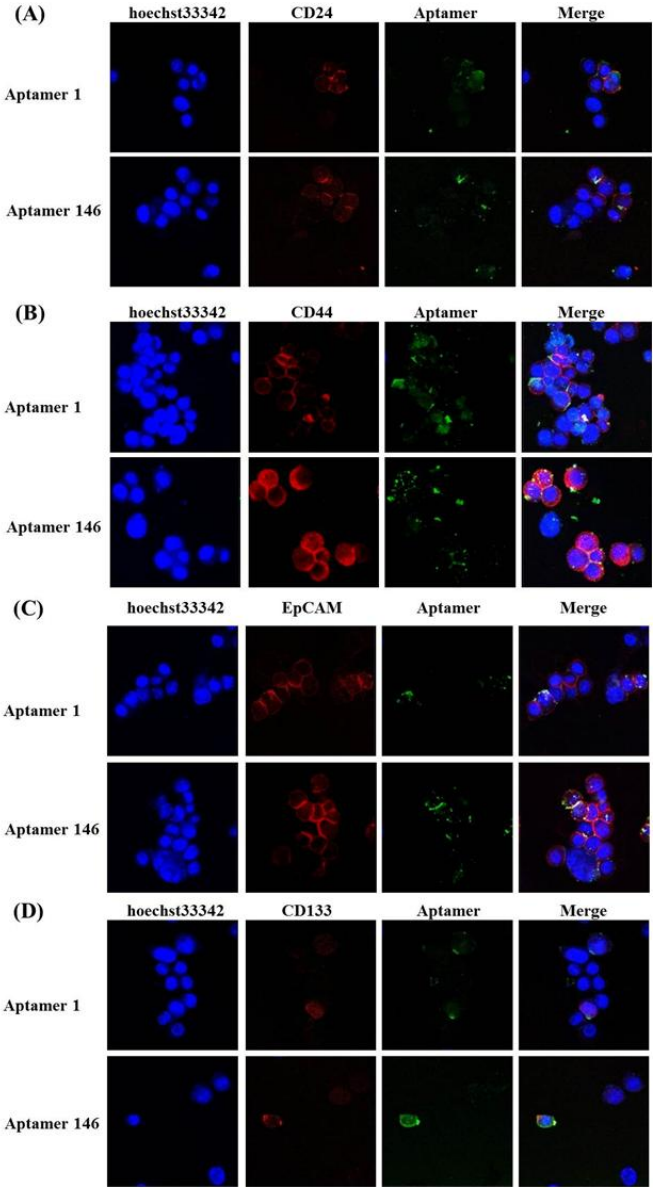

Figure 5. Co-localization of CSC markers were observed in the aptamer-positive cells by confocal microscopy. HPAC cells were incubated with FITC-labeled aptamer, PE-conjugated CSC markers, and Hoechst dye, which was used to visualize the cell nucleus. Cells that highly expressed CSC markers were observed and these cells were found to show significant co-localization with fluorescence generated by binding of aptamer 1 or 146 to the cell surface. (A: CD24, B: CD44, C: EpCAM, D: CD133, ×40)

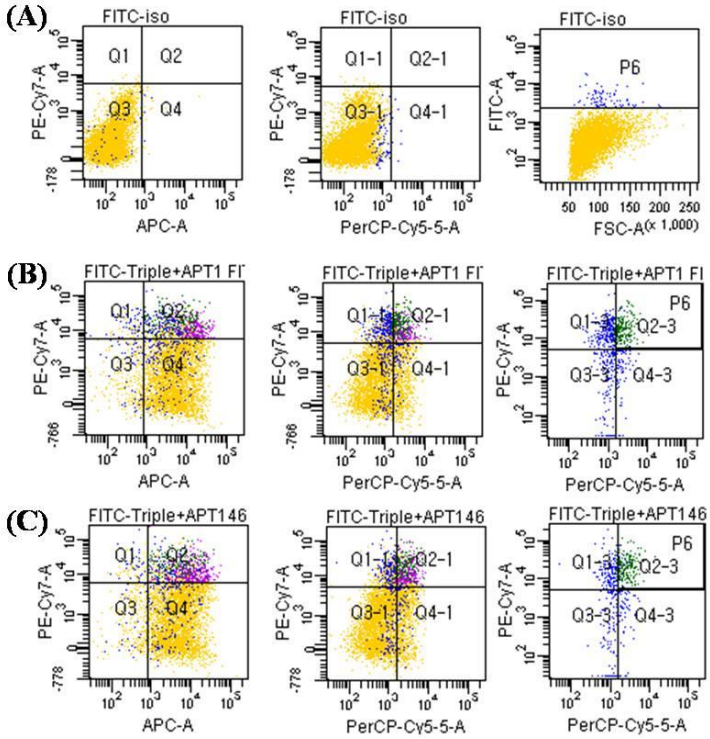

Figure 6. The relationship between CSC triple positive cells and aptamers by flow cytometry. In triple positive cells, we found a small population of cells in the total HPAC cell lines. However, the proportion of triple-positive cells was enriched in aptamer-positive population (aptamer 1: 60%, aptamer 146: 67%). (APC-CD44, PE-Cy7-CD24, PerCP-Cy5.5-ESA, FITC-aptamer 1 or aptamer 146) (A: control, B: triple marker + aptamer 1, C: triple marker + aptamer 146)

6. Sphere forming assay

Sphere formation is a common characteristic of stem cells. To evaluate aptamer 1 as a candidate CSC-associated marker, spheres established from aptamer 1-positive or -negative cells

As a result, aptamer 1-positive cells generated spheres more than aptamer 1-negative cells (Figure 7).

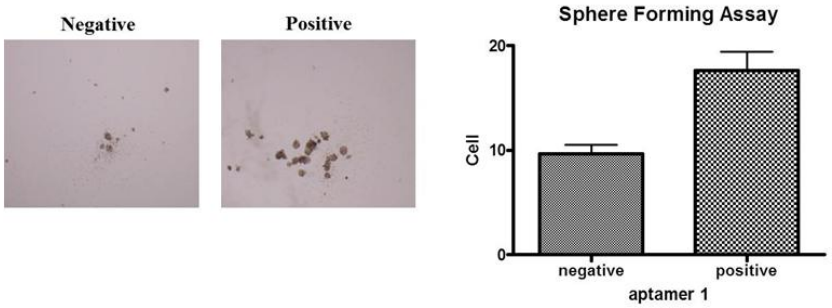

Figure 7. sphere forming ability of aptamer dependent cells. Spheroid cells established from aptamer 1-positive and -negative cells. The aptamer 1-positive cells were more generative of spheres than the aptamer 1-negative cells ($p < 0.05$).

7. Circulating tumor cell detection assay

Recently, it is published CSC markers are frequently over-expressed in the CTCs of patients with metastatic breast cancer.[12] Also, in another report, the detection of CSCs and CTCs in tumor drainage vein blood samples has prognostic significance in patients with Duke's stage Band C colorectal cancer.[13]

In our data, we confirmed that the aptamer 1-positive cells had different expression levels of CSC- and EMT-related genes and generated more spheres than aptamer 1-negative cells.

Therefore, we bound aptamer 1 to human PDAC-derived CTCs with cytokeratin and CD133. CD133, a pancreatic-CSC marker, was also highly expressed in aptamer-positive cells.

After passing blood samples through the OncoQuick tubes, collected cells were incubated with DAPI, cy5-labeled aptamer 1, FITC-conjugated cytokeratin, and PE-conjugated CD133. Figure 8 shows fluorescent images of cells isolated from blood samples from metastatic pancreatic cancer patients. As expected, CTCs were detected using CSC markers, such as CD133 with aptamer1. Four of five metastatic pancreatic cancer patients had more than eight CD133+/aptamer 1+ CTCs.

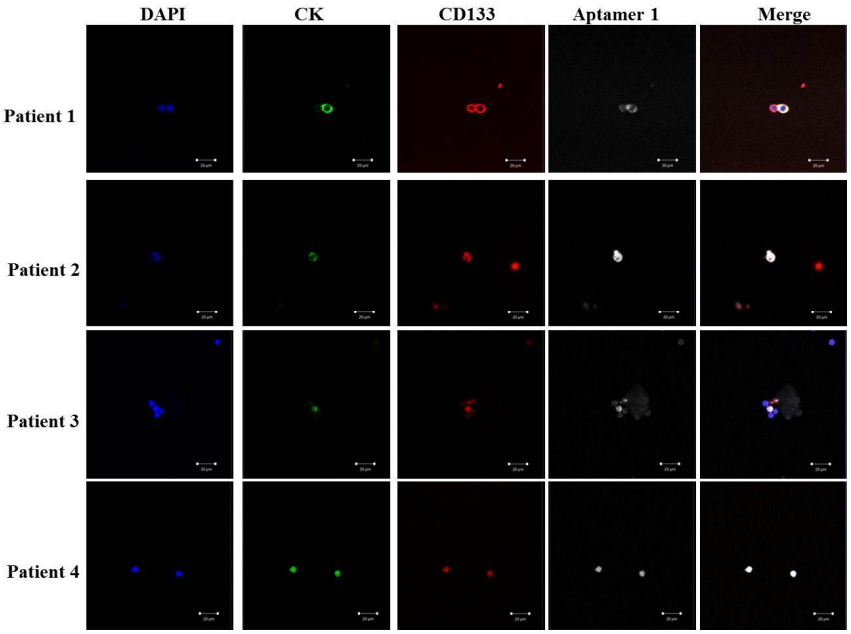

Figure 8. Confocal images of Circulating Tumor Cell (CTC) detection in human blood. Human PDAC-derived CTCs were also incubated with FITC-conjugated cytokeratin, PE-conjugated CSC markers, cy5-labeled aptamer 1, and Hoechst dye, which was used to visualize the cell nucleus. (A: EpCAM, B: CD24, C: CD133, 40X)

IV. DISCUSSION

Despite multiple clinical trials and continuous efforts, pancreatic cancer remains the most difficult cancers to cure because of its resistance to conventional chemotherapy and difficulty of early diagnosis. Recent reports have attempt to solve these problem by targeting CSC, unfortunately, is still unanswered. As for pancreatic CSCs, CXCR4/CD133 and CD44/CD24/EpCAM have been reported as being selective to pancreatic CSCs. However, further research is necessary to confirm that these genes act as diagnostic markers of pancreatic CSCs, as these multiple markers must be combined.

Targeting a protein or a cell surface, the aptamers can be used as diagnostic markers in cancer research. Aptamers targeting CSC-specific surface markers such as ErbB2[2] or EpCAM[6] have been generated; however, neither are absolute pancreatic CSC markers.

To overcome this situation, we decided to develop the pancreatic CSC-associated aptamers using modified Cell-SELEX method. Also, we designed the DNA aptamers targeted for stemness-enriched cancer cells in pancreatic cancer without multiple markers; thus, it is more simple and convenient to evolve CSC targeting molecules. To generate pancreatic CSC-associated aptamer, we enriched stemness from HPAC human pancreatic cancer cell lines using sphere formation assay.[14] Previously, we have observed that the sphere formation generated by HPAC cell line, possesses the stem cell-like characters by expressing stem cells-associated genes including oct4, nanog, and Hedgehogs as a marker in

dependent method. After cell-SELEX process, 7 individual sequences were selected with no similarity. Among sorted 7 sequences, aptamer 1 and aptamer 146 were highly affinitive to stemness-enriched cancer cells in pancreatic cancer, and selective to stemness-enriched cancer cells in pancreatic cancer than pancreatic normal cell line and hMSC. Particularly, aptamer-positive cells were found to be over-expressed CSC-related genes. Also, aptamer 1 and aptamer 146 are co-localized with existing CSC markers such as CD44, CD24, EpCAM and CD133.

For the further application, we examined CSC marker enriched CTC detection assay. Some reports have suggested CSC markers as CTC detection marker, but not solid in pancreatic cancer.[12, 13] In this situation, aptamer 1 would be able to cover to various existing CSC markers. Thus, these findings suggest that aptamer 1 is a potential candidate for CSC-enriched CTC detection in cases of pancreatic cancer.

In summary, our results demonstrated that the aptamer 1 and aptamer 146 are highly affinitive and specific to stemness-enriched cancer cells in pancreatic cancer. And we verified aptamer 1 and aptamer 146 are co-localized with CSC markers such as CD44, CD24, ESA and CD133. Also, we performed qRT-PCR assay to confirm the expression level of CSCs-related genes.

Furthermore, aptamer 1 recognized CSC marker-enriched CTCs in cases of pancreatic cancer, suggesting that aptamer 1 may play the roles of a diagnostic marker and therapeutic agent.

V. CONCLUSION

In conclusion, pancreatic CTC specific aptamers appear to be an excellent diagnostic marker in pancreatic cancer patients. The present data warrant further clinical investigation to demonstrate the impact of pancreatic CTC specific aptamers. In the future, the test using pancreatic CSC-associated aptamers may diagnose more patients with pancreatic cancer, some of them at an early stage. Therefore, this approach has a potential to increase the number of patients that can be operated on and possibly cured of pancreatic cancer.

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ABSTRACT(IN KOREAN)

췌장암에서 암줄기세포 특이적 앱타머의 개발

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김 윤 진

췌장암은 가장 치명적인 암 중 하나이다. 대부분의 췌장암 환자(80%)는 처음 진단시 수술로 치료할 수 없을 정도로 진행된 상태로 발견되기 때문에 조기진단이 시급히 요망된다. 최근 연구에 따르면 췌장암을 포함한 대부분의 암은 암줄기세포에서 시작되고 퍼지는 것으로 알려졌다. 이러한 암줄기세포는 암의 발병 뿐 아니라 이형성, 악성의 정도, 항암치료 내성과 재발에 관여하는 것으로 보고된 바 있다. 그러므로 바이오마커를 통한 암줄기세포의 동정은 치명적인 췌장암을 극복할 수 있는 디딤돌이 될 수 있다.

여기서 우리는 췌장암 특이 앱타머를 진단과 치료의 도구로서 제안하고자 한다. 췌장암 암줄기세포에 특이적인 앱타머는 변형된 cell-SELEX 방법을 동해서 개발하였다. 양성 선별은 HPAC 이라는 췌장암 세포주에서부터 만들어진 sphere 세포로 수행하였고 그 후, HPDE라는 정상 췌장 세포주로 음성 선별을 수행하였다. cell-SELEX 이후, 앱타머 1과 146이 해리정수값 22.18 nM에서 22.62 nM로 가장 높은 친화력을 보였다. 이 두 앱타머는 sphere 세포에는 붙고 정상세포주인 HPDE에 붙지 않아

췌장암 암세포주에만 선택성을 가지는 것으로 확인되었다.

앱타머를 HPAC세포주에 결합시켰을 때 앱타머 1 또는 146이 붙은 앱타머 양성 세포는 암줄기세포 관련 유전자가 앱타머가 붙지 않은 앱타머 음성 세포에 비하여 높은 발현 수준을 보였다. 또한 앱타머 1 또는 146은 HPAC 세포에서 CD44, CD24, ESA 그리고 CD133과 같은 췌장암 암줄기세포 관련 표적 마커와 공동으로 위치함을 형광현미경으로 확인하였다.

본 연구에서는 이 두 췌장암 암줄기세포 특이 앱타머가 새로운 진단 마커, 약물 전달을 위한 표적 후보 또는 순환 종양 세포의 검출을 위한 잠재적인 마커가 될 가능성을 규명했다.

핵심되는 말 : 췌장암, 암줄기세포, 앱타머

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