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Claudin 7 as a possible novel molecular target for the treatment of pancreatic cancer

Norimitsu Okui [a], Yuko Kamata [b], Yukiko Sagawa [b], Akiko Kuhara [b], Kazumi Hayashi [c], Tadashi Uwagawa [a], Sadamu Homma [b, *], Katsuhiko Yanaga [a]

[a] Department of Surgery, The Jikei University School of Medicine, Tokyo, Japan
[b] Division of Oncology, Research Center for Medical Sciences, The Jikei University School of Medicine, Tokyo, Japan
[c] Division of Clinical Oncology and Hematology, Department of Internal Medicine, The Jikei University School of Medicine, Tokyo, Japan

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ABSTRACT

Background/objectives: Pancreatic cancer consists of various subpopulations of cells, some of which have aggressive proliferative properties. The molecules responsible for the aggressive proliferation of pancreatic cancer may become molecular targets for the therapies against pancreatic cancer.
Methods: From a human pancreatic cancer cell line, MIA PaCa-2, MIA PaCa-2-A cells with an epithelial morphology and MIA PaCa-2-R cells with a non-epithelial morphology were clonogenically isolated by the limiting dilution method. Gene expression of these subpopulations was analyzed by DNA microarray. Gene knockdown was performed using siRNA.
Results: Although the MIA PaCa-2-A and MIA PaCa-2-R cells displayed the same DNA short tandem repeat (STR) pattern identical to that of the parental MIA PaCa-2 cells, the MIA PaCa-2-A cells were more proliferative than the MIA PaCa-2-R cells both in culture and in tumor xenografts generated in immunodeficient mice. Furthermore, the MIA PaCa-2-A cells were more resistant to gemcitabine than the MIA PaCa-2-R cells. DNA microarray analysis revealed a high expression of claudin (CLDN) 7 in the MIA PaCa-2-A cells, as opposed to a low expression in the MIA PaCa-2-R cells. The knockdown of CLDN7 in the MIA PaCa-2-A cells induced a marked inhibition of proliferation. The MIA PaCa-2-A cells in which CLDN7 was knocked down exhibited a decreased expression of phosphorylated extracellular signal-regulated kinase (p-Erk)1/2 and G1 cell cycle arrest.
Conclusions: CLDN7 may be expressed in the rapidly proliferating and dominant cell population in human pancreatic cancer tissues and may be a novel molecular target for the treatment of pancreatic cancer.

Introduction

Pancreatic cancer is a common cause of cancer-related mortality worldwide [1,2]. This disease is so aggressive that the tumors of the majority of patients are unresectable at the time of diagnosis. Although 10% of patients can be treated with surgical resection, >80% relapse within 2 years [3]. The median survival is < 1 year for patients with locally advanced disease and 3–6 months for patients with metastases [4]. Chemotherapy is used to prolong the survival of patients with unresectable pancreatic cancer [5,6]. Gemcitabine has been the standard treatment regimen and is widely used in pancreatic cancer therapy [7]. The newer regimens, FOLFIRINOX (combination of oxaliplatin, irinotecan, fluorouracil and leucovorin) and gemcitabine/nab-paclitaxel have led to improved survival compared to treatment with gemcitabine alone and are now becoming standard treatments in developed countries [8,9]. However, as these therapies have considerably limited effects on advanced pancreatic cancer, the establishment of effective therapies against pancreatic cancer is urgently required.

The understanding of the heterogeneity of tumor cells composing the tumor tissue is of great importance for the treatment of malignant tumors [10]. Populations of tumor cells with different phenotypic characteristics exist within the tumor mass, and they are sometimes diffusely combined or intermingled [11,12].

* Corresponding author. Division of Oncology, Research Center for Medical Sciences, The Jikei University School of Medicine, 3-25-8 Nishi-shimbashi, Minato-ku, Tokyo, 105-8461, Japan.
E-mail address: sahya@jikei.ac.jp (S. Homma).

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Different subpopulations of tumor cells in the tumor tissue provide different sensitivities to therapeutic agents, leading to the selection of surviving cells that are resistant to drugs [13,14]. Moreover, epithelial-mesenchymal transition can also generate many variants of tumor cells [15,16]. If the cancer stem cell theory is applicable for the development of malignant tumors, it would indicate that the tumor cells with variable phenotypes can differentiate from the cancer stem cells through non-symmetrical cell division [17,18].

Although *Kras* mutations are a pivotal factor for the development of pancreatic cancer [19], the heterogeneity of pancreatic cancer cells with different phenotypes has also been reported [20]. If pancreatic cancer tissues contain a clonal subpopulation of cells that are highly proliferative, these cells would dominate the tumor, resulting in aggressive disease. The identification of genes responsible for conferring such malignant phenotypes is essential because the inhibitors of these gene products can become promising therapeutic modalities for molecular targeted therapy.

CLDN family are important components of tight junctions regulating paracellular permeability, cell polarity and barrier function permanence [21,22], and are closely associated with malignant phenotypes [23,24]. CLDN-associated cell-to-cell adhesion controlling luminal barrier, paracellular transport and signal transduction is an important factor for the proliferation, transformation and metastasis of tumor cells [23]. Although the expression of CLDN in human pancreatic cancer tissue has been widely investigated [25–28], the involvement of CLDN7 in human pancreatic cancer has not yet been defined [29–32].

In this study, we isolated several clonal populations representing aggressive and non-aggressive phenotypes from a human pancreatic cancer cell line and subsequently identified CLDN7 as a gene specifically expressed in the clonal population with aggressive phenotypes.

Methods

Cell culture

The human pancreatic adenocarcinoma cell line, MIA PaCa-2, was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The MIA PaCa-2 cells were maintained in Dulbecco's modified Eagle's medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (Biosera, Kansas City, MO, USA) and penicillin/streptomycin/Amphotericin B (Nacalai Tesque). The cells were cultured at 37 °C in a fully humidified atmosphere with 5% CO_2. Other human pancreatic cancer cell lines, namely Panc-1, BxPC, AsPC, Capan-1 and Capan-2, and a human breast cancer cell line, MCF7 (positive control for CLDN7 expression), were purchased from ATCC.

Isolation of several cell subpopulations derived from single MIA PaCa-2 cells

The limiting dilution method was used for the isolation of single cell clones from the MIA PaCa-2 cells. Single MIA PaCa-2 cells were placed in wells of culture plates, and the clonal populations growing from each single cell were isolated. Subpopulations of cells showing epithelial or non-epithelial like morphology were isolated, and single cell-derived clones were again generated from these subpopulations.

DNA short tandem repeat (STR) analysis

DNA STR analysis was performed at the JCRB Cell Bank of the National Institute of Biomedical Innovation (Osaka, Japan). The data from the DNA STR analysis of the MIA PaCa-2-A cells and MIA PaCa-2-R cells were compared with those of the MIA PaCa-2 cells registered in ATCC (CRL-1420 MIA PaCa-2) and JCRB (JCRB0070 MIA PaCa-2).

Western blot analysis

The cells were lysed in RIPA buffer (Nacalai Tesque) supplemented with phosphatase inhibitor cocktail (Nacalai Tesque) and protease inhibitor cocktail for general use (Nacalai Tesque). In total 20 or 15 μg of protein were separated on 5–20% gradient e-PAGEL gels (ATTO Corp., Tokyo, Japan). The proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes using the iBlot transfer system (Thermo Fisher Scientific, Waltham, MA, USA) or Trans-Blot Turbo system (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with Blocking One-P buffer (Nacalai Tesque) for 20 or 30 min or 1 h at room temperature for detecting phosphoproteins. The membranes were then blocked with Blocking One buffer (Nacalai Tesque) for 1 h at room temperature to detect other proteins. The antibodies used for western blot analysis are shown in Supplement Methods. The membranes were incubated with the corresponding primary antibodies in TBST (0.01 M Tris-HCl and 0.15 M NaCl with 0.01% Tween-20) buffer with 5% Blocking One-P and phosphatase inhibitor cocktail (1:1000, Nacalai Tesque) for phosphoprotein detection or in phosphate-buffered saline (PBS) with 0.1% Tween-20 (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4) buffer with 5% Blocking One buffer for other protein detection with gentle agitation overnight at 4 °C followed by incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (GE Healthcare UK.Ltd. Little Chalfont, Backinghamshire, UK) or HRP-conjugated anti-mouse IgG (GE Healthcare UK Ttd.) for 1 h at room temperature. The signal was visualized using Chemi-Lumi One Super (Nacalai Tesque).

Cell proliferation assay

For the evaluation of cell proliferation *in vitro*, the cells (5×10^2 cells/well) were seeded in 96-well culture plates. Cell proliferation was determined at 24, 72, 120 and 168 h after plating by spectrophotometry using Cell Count Reagent SF (Nacalai Tesque). Fluorescence was measured at 450 nm using an iMark microplate absorbance reader (Bio-Rad Laboratories). For the evaluation of the antitumor effect of gemcitabine, cells (5×10^3 cells/well) were seeded in 96-well culture plates. Following incubation for 24 h, the cells were treated with gemcitabine (Eli Lilly Japan, Kobe, Japan) at 0–1000 ng/ml for 72 h. Cell proliferation was determined with Cell Count Reagent SF.

Tumor formation in severe combined immunodeficiency (SCID) mice by the inoculation of tumor cells

The inoculation of tumor cells was performed using a suitable mouse restrainer to minimize the suffering of the animals. SCID female mice were purchased from Nihon SCL Co., Ltd. (Hamamatsu, Japan), and separated into 3 groups, 6 mice in each. Parental MIA PaCa-2, MIA PaCa-2-A or MIA PaCa-2-R cells (10^6 cells/mouse) were inoculated subcutaneously into the mice in each group, respectively. The mice were sacrificed by intra-peritoneal injection of pentobarbital when the longest tumor diameter reached to 20 mm. All animal studies were conducted according to the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Jikei University (Permit no. 26-018) and followed the Animal Research: Reporting of in Vivo Experiments (ARRIVE) guidelines.

DNA microarray analysis

Microarray analysis was performed by TransGenic Inc. (Kumamoto, Japan). Total RNA was extracted from the MIA PaCa-2-A and MIA PaCa-2-R cells. The RNA integrity number (RIN) of the total RNA was determined using a BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). Total RNA (RIN $\geq$8.0) was used for microarray analysis. cDNA synthesis was performed with a TargetAmp-Nano Labeling kit for Illumina Expression BeadChip (Illumina, San Diego, CA, USA). Hybridization of the cDNA to HumanHT-12 v4 BeadChip (Illumina), washing and staining were performed using the Illumina Gene Expression system Hyb E1 and a Blocking Buffer kit (Illumina), with a Wash E1BC Buffer kit and a High Temp Wash Buffer kit (Illumina) and with Streptavidin-Cy3 (GE Healthcare UK Ltd, Buckinghamshire, England), respectively. The BeadChip was then scanned with a BeadStation 500GX-WG system (Illumina) and analyzed using GenomeStudio (Illumina), GeneSpringGX 12.6 (Agilent Technologies) and GeneViewer 2.01 (TransGenic Inc., Fukuoka, Japan) software.

Knockdown of CLDN7 gene by siRNA

The cells were seeded in 6 or 10-cm Petri dishes (Corning Inc., Corning, NY, USA or TPP Techno Plastic Products, Trasadingen, Switzerland). Following overnight incubation, siRNA specific for CLDN7 or negative control siRNA (Sigma-Aldrich, St. Louis, MO, USA) transfection was performed using Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to manufacturer's instructions and further incubated for 24 or 48 h.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using an RNeasy Plus Mini kit (Qiagen GmbH, Hilden, Germany). cDNA was synthesized from 500 ng of total RNA using a PrimeScript RT Reagent kit (Takara Bio, Shiga, Japan) and the GeneAmp PCR System 9700 (Thermo Fisher Scientific). For RT-PCR detection of CLDN7 and 18S rRNA, 100 ng of cDNA was amplified using TaqMan Universal Master Mix II, no UNG (Thermo Fisher Scientific) with the 7300 Real-Time PCR System (Thermo Fisher Scientific). The PCR conditions consisted of an initial denaturation step (95 °C for 10 min) followed by 40 cycles (95 °C for 15 s and 60 °C for 1 min). The TaqMan primers for the CLDN7 (Assay ID: Hs00600772_m1) and 18S ribosomal RNA (Assay ID: Hs99999901_s1) genes were purchased from Thermo Fisher Scientific. Relative expression was calculated using the delta-delta Ct method [33].

Cell cycle analysis

The MIA PaCa-2-A cells were untreated or transfected with CLDN7-specific siRNA or control siRNA. After 24 h incubation, untreated or siRNA-transfected cells were synchronized by 48 h serum starvation. The cells were then detached by trypsin-EDTA traetment and further incubated in the complete medium without siRNA.The cells were harvested immediately after the serum starvation and after following 24 h incubation. They were fixed with 70% (v/v) ethanol and stored at −20 °C until use. Following centrifugation, the cell pellet was washed twice with PBS, re-suspended in PI/RNase Staining Buffer (BD Biosciences, Franklin Lakes, NJ, USA), and incubated at room temperature for 15 min. The cells were then analyzed using a MACSQuant Analyzer (Miltenyi Biotec K.K., Bergisch Gladbach, Germany).

Statistical analysis

Data are expressed as the means values ± standard deviation. A non-paired *t*-test and repeated measures analysis of variance were used for statistical analyses. All P-values were considered to indicate statistically significant differences when the associated probability was <0.05.

Results

Isolation of clonal cells with high and low malignancy phenotypes from MIA PaCa-2 cells

Under a phase contrast microscope, the MIA PaCa-2 cells consisted of a mixture of large epithelial-like cells that firmly attached to the plastic dish and small non-epithelial-like cells that were loosely attached to the plastic dish (Fig. 1A). Following single cell cloning by the repeated limited dilution method, clonal cell populations with each morphological characteristic could be isolated; 7 epithelial-like clones and 9 non-epithelial-like clones were obtained. A representative population of clonal cells exhibiting an epithelial-like morphology was designated as the MIA PaCa-2-A cells, while the population of cells exhibiting a non-epithelial-like morphology was designated as the MIA PaCa-2-R cells (Fig. 1A). The letter 'A' in MIA PaCa-2-A indicates 'adhesive' and 'R' in MIA Paca-2-R indicates 'round'. The images in the upper panels in Fig. 1A show the MIA PaCa-2-A and MIA PaCa-2-R cells directly after establishment, and the images on the lower panels show each cell population maintained for >4 years, showing that each clonal population maintained its morphological characteristics and did not exhibit any change in morphology even after long-term culture. DNA STR analysis demonstrated that the MIA PaCa-2-A and MIA PaCa-2-R cells had the same pattern of DNA STR identical to that of the parental MIA PaCa-2 cells (Supplement Table 1 and Fig. 1B), indicating that the MIA PaCa-2-A and MIA PaCa-2-R cells were derived from the MIA PaCa-2 cells and were not contaminated cells by other cell lines. The activation of Erk1/2, Akt, NF-κB and STAT3 pathways is closely associated with the morphogenesis, proliferation, survival and drug resistance of malignant cells [34–36]. A higher expression of p-NF-κB was observed in the MIA PaCa-2-A cells compared with the MIA PaCa-2-R cells, while p-STAT3 expression was observed in the MIA PaCa-2-R cells, but not in the MIA PaCa-2-A cells (Fig. 1C). The expression of p-Erk1/2 was similar between the MIA PaCa-2-A and MIA PaCa-2-R cells, and the expression of p-Akt was not seen in both the MIA PaCa-2-A and MIA PaCa-2-R cells.

The expression of molecules related to epithelial-mesenchymal transition (EMT) was examined in the MIA PaCa-2-A and MIA PaCa-2-R cells by DNA microarray (Supplement Table 2). CDH1, an epithelial marker, was expressed in the MIA PaCa-2-A cells, but not in the MIA PaCa-2-R cells. However, the expression of mesenchymal markers and cancer stem-like cell markers did not differ between the MIA PaCa-2-A and MIA PaCa-2-R cells.

The MIA PaCa-2-A cells were more proliferative than the MIA PaCa-2-R cells (Fig. 2A). The inoculation of MIA PaCa-2-A cells into SCID mice produced rapidly growing tumors; however, the inoculation of the MIA PaCa-2-R cells produced tumors that grew at a significantly slower rate (Fig. 2B). Furthermore, the MIA PaCa-2-A cells were more resistant to gemcitabine, a standard chemotherapeutic drug used in the treatment of pancreatic cancer, than the MIA PaCa-2-R cells (Fig. 2C). These results indicate that the MIA PaCa-2-A and MIA PaCa-2-R cells represent populations with high and low malignant characteristics, respectively.

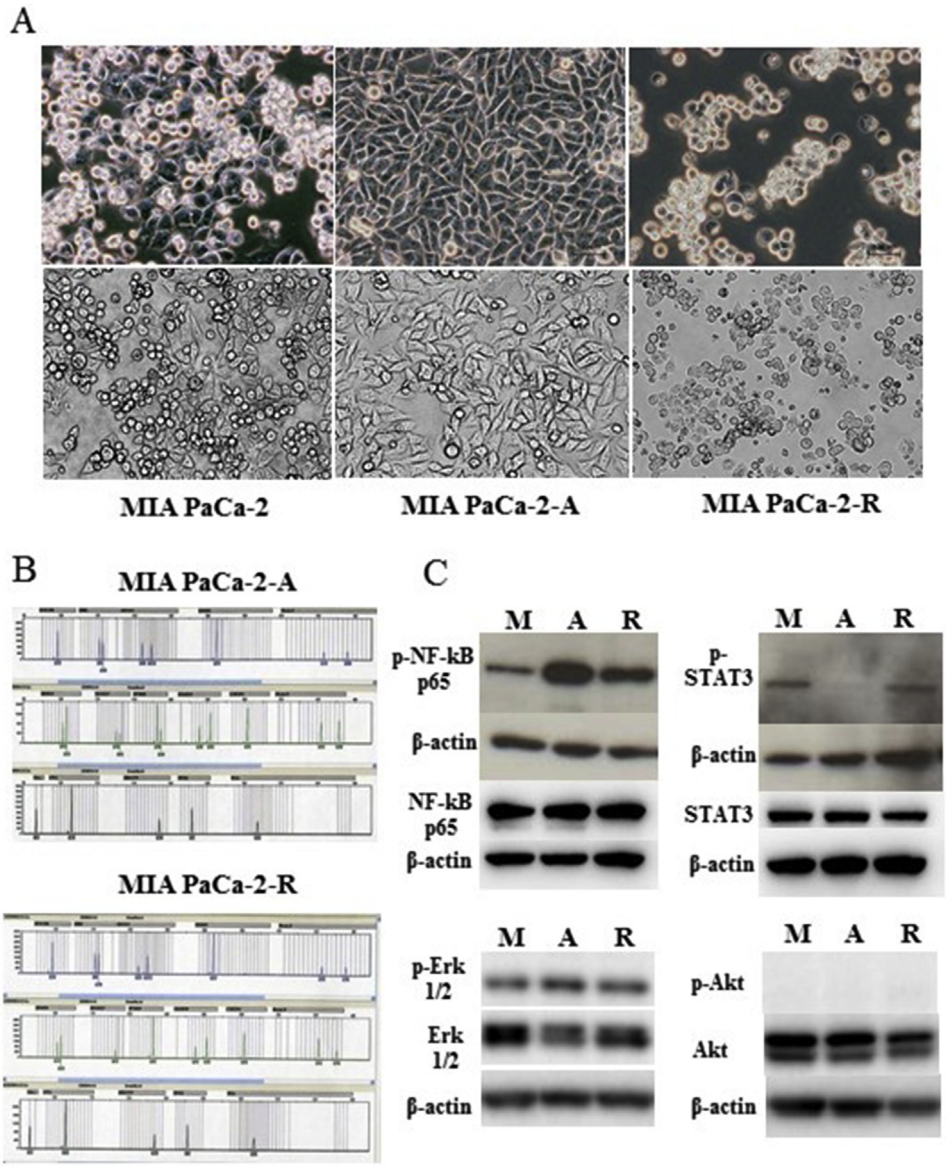

Fig. 1. Morphologically and functionally distinct clonal populations were isolated from the MIA PaCa-2 cell line. (A) Left panels, MIA PaCa-2 cells; middle panels, MIA PaCa-2-A cells isolated by the limited dilution of MIA PaCa-2 cells; right panels, MIA PaCa-2-R cells isolated by the limited dilution of MIA PaCa-2 cells; upper panels: MIA PaCa-2-A and MIA PaCa-2-R cells directly after establishment; lower panels, MIA PaCa-2-A and MIA PaCa-2-R cells after maintenance for >4 years. (B) DNA short tandem repeat (STR) patterns of the MIA PaCa-2-A and MIA PaCa-2-R cells. (C) Western blot analysis of cell signaling pathways. M, MIA PaCa-2 cells (mixture of epithelial-like cells and non-epithelial-like cells); A, MIA PaCa-2-A cells (epithelial-like cells); R, MIA PaCa-2-R cells (non-epithelial-like cells). p-, phosphorylated.

CLDN7 expression is high in the MIA PaCa-2-A cells, but low in the MIA PaCa-2-R cells

DNA microarray analysis demonstrated that the expression of several genes differed markedly between the MIA PaCa-2-A and MIA PaCa-2-R cells (Fig. 3A). The genes that were expressed in the MIA PaCa-2-A cells, but not in the MIA PaCa-2-R cells and corresponded to the Gene Ontology process of 'adhesion' were selected as the MIA PaCa-2-A-specific adhesion-related genes and are listed in Table 1. According to the difference in the morphology between the MIA PaCa-2-A cells and MIA PaCa-2-R cells, the expression of molecules composing tight junctions is also considered to differ [37]. As CLDN was the gene mostly associated with tight junctions among the molecules listed in Table 1 [38], we focused CLDN 7 as a target molecule. Furthermore, CLDN 7 exhibited the greatest difference in expression between the MIA PaCa-2-A and MIA PaCa-2-R cells among the CLDN family members. (Supplement Table 3). RT-PCR analysis of CLDN7 expression also revealed a higher CLDN7 expression in the MIA PaCa-2-A cells compared with the MIA PaCa-2-R cells (Fig. 3B). Western blot analysis demonstrated that CLDN7 protein expression was also high in the MIA PaCa-2-A cells, but low in the MIA PaCa-2-R cells (Fig. 3C). CLDN7 protein expression was examined in several human pancreatic cancer cell lines other than the MIA PaCa-2, MIA PaCa-2-A and MIA PaCa-2-R cells. Human pancreatic cancer cell lines, Panc-1, BxPC, ASPC, Capan-1 and Capan-2, also expressed CDLN7 by western blot analysis (Fig. 3C).

Knockdown of CLDN7 gene in the MIA PaCa-2-A cells suppresses proliferation

Treatment of the MIA PaCa-2-A cells with siRNA specific to CLDN7 decreased the protein and mRNA expression of CLDN7

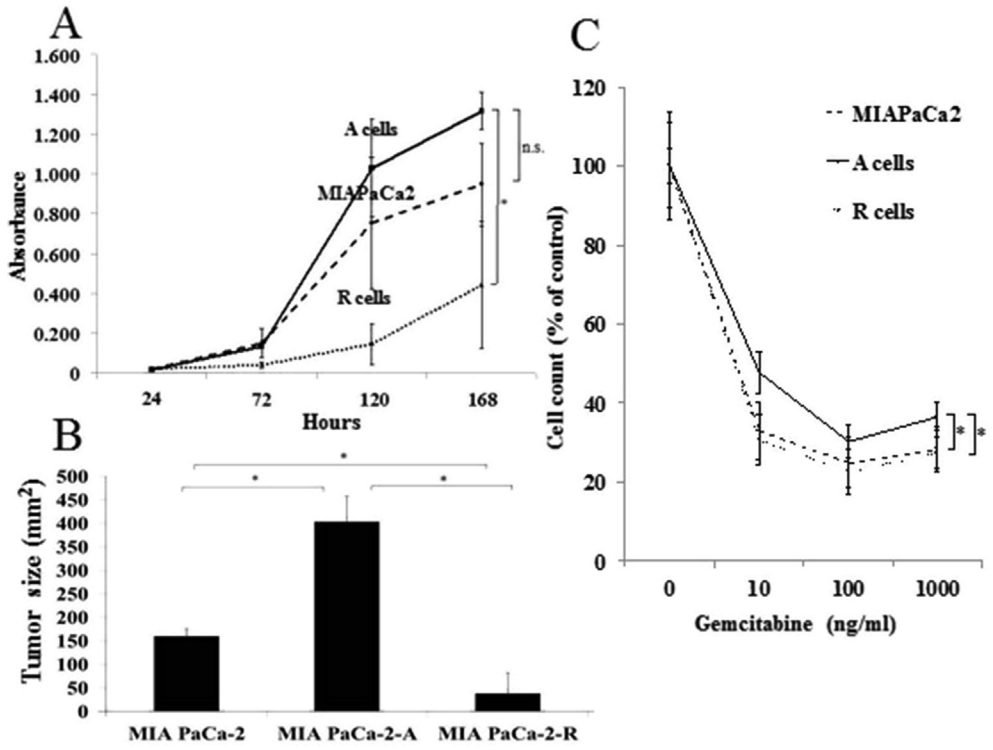

Fig. 2. Different proliferative capacities and drug sensitivities of the MIA PaCa-2, MIA PaCa-2-A and MIA PaCa-2-R cells. (A) The MIA PaCa-2, MIA PaCa-2-A or MIA PaCa-2-R cells were seeded in 96-well culture plates (5×10^2 cells/well). Following incubation for 48, 72, 120 and 168 h, cell numbers were determined using Cell Count Reagent SF. The results are presented as the mean values from 3 independent experiments, and the bars indicate standard deviation; $^*p < 0.01$; n.s., not significant. (B) The MIA PaCa-2 cells, MIA PaCa-2-A or MIA PaCa-2-R cells were subcutaneously inoculated into SCID mice (10^6 cells/mouse, $n = 6$). Tumor size (long diameter x short diameter, mm^2) was determined 4 weeks after the inoculation by measuring each tumor with a caliper. The same experiment was repeated twice, and similar results were obtained; $^*p < 0.01$. (C) The MIA PaCa-2 cells, MIA PaCa-2-A or MIA PaCa-2-R cells were seeded in 96-well culture plates (5×10^3/well). Following 24 h of incubation, the cells were untreated or treated with gemcitabine at indicated concentrations for 72 h. The cell number was then determined using Cell Count Reagent SF. The number of viable cells after each treatment was expressed as a ratio to that of untreated cells. The results are shown as the average of those in 3 independent experiments and the bars indicate standard deviation; $^*p < 0.01$.

(Fig. 4A and B). Knockdown of the CLDN7 gene in the MIA PaCa-2-A cells significantly suppressed proliferation (Fig. 4C). The morphology of the MIA PaCa-2-A cells in which CLDN7 was knocked down did not differ significantly and was almost the same as that of the control siRNA-transected MIA PaCa-2-A cells (Fig. 4D). The expression of p-Erk1/2 in the MIA PaCa-2-A cells was suppressed by transfection with CLDN7 siRNA (Fig. 4E). The activation status of NF-κB in the MIA PaCa-2-A cells was not markedly affected by transfection with CLDN7 siRNA (Fig. 4E).

CLDN7 knockdown induces G1 cell cycle arrest

The cell cycle distribution of the CLDN7-specific siRNA-transfected MIA PaCa-2-A cells was examined by flow cytometry. After 48 h serum starvation, a higher frequency of cells at the G1 phase and a lower frequency of cells at the G2 phase were observed in the MIA PaCa-2-A cells transfected with CLDN7-specific siRNA (Fig. 5C) than in those untreated or transfected with control siRNA (Fig. 5A and B). Following incubation for 24 h, the frequency of cells in the G1 phase increased in the CLDN7 siRNA-transfected cells (Fig. 5F) compared with the untreated or control siRNA-transfected cells (Fig. 5D and E). These results indicate that G1 arrest and the resultant G1/S block were induced by the knockdown of CLDN7.

Discussion

In this study, we isolated morphologically different subpopulations derived from single MIA PaCa-2 cells, namely MIA PaCa-2-A cells with an epithelial morphology and MIA PaCa-2-R cells with a non-epithelial morphology. The MIA PaCa-2-A and MIA PaCa-2-R cells had the same DNA STR pattern as the parental MIA PaCa-2 cells, indicating that they were not contaminants derived from other cell lines. Apart from our isolation, Walsh et al. [39] described that several subpopulations of cells with different morphologies were isolated from MIA PaCa-2 cells. Each subpopulation exhibited different capacities for invasion, adhesion, anoikis and anchorage-independent growth, and these functional differences were associated with the types of integrins expressed by each subpopulation [39]. According to the results of the *in vivo* and *in vitro* proliferation studies and gemcitabine sensitivity, the MIA PaCa-2-A and MIA PaCa-2-R cells represent populations of the MIA PaCa-2 cells with high and low malignant phenotypes, respectively.

DNA microarray analysis demonstrated that CLDN7 was highly expressed in the MIA PaCa-2-A cells, but not in the MIA PaCa-2-R cells. The CLDN family has 27 members and shows specific distribution. Proteins of the CLDN family are important components of tight junctions regulating paracellular permeability, cell polarity and barrier function permanence [21,22]. CLDN7 has been reported as one of the impermeable CLDNs and interacts directly with epithelial cell adhesion molecule (EpCAM) to strengthen the junction of epithelial cells [40]. CLDN-associated cell-to-cell adhesion is an important factor for the proliferation, transformation and metastasis of tumor cells [23]. It is conceivable that the MIA PaCa-2-A cells showing tight epithelial feature express high levels of CLDN7 and that the MIA PaCa-2-R cells showing round shape and loosely attached features do not.

It has been reported that the expression of CLDN family proteins is deregulated and is closely associated with malignant phenotypes

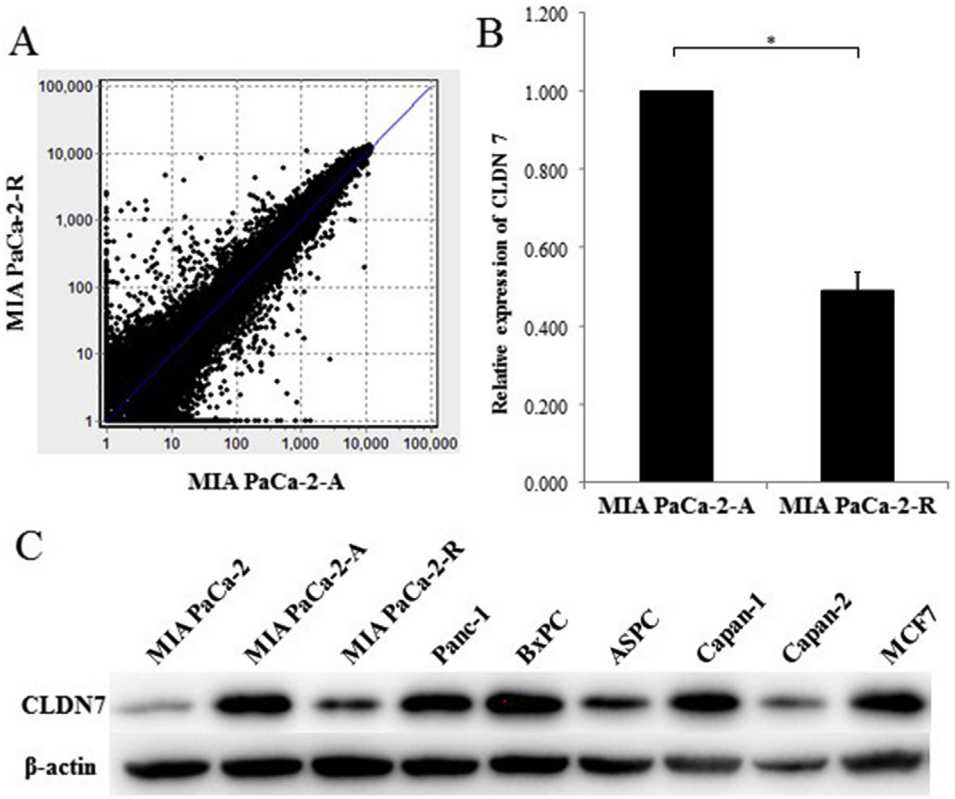

Fig. 3. Different gene expression profiles between the MIA PaCa-2-A and MIA PaCa-2-R cells. (A) A scatter plot of the gene expression of the MIA PaCa-2-A and MIA PaCa-2-R cells by DNA microarray analysis. The vertical axis indicates the intensity of gene expression in the MIA PaCa-2-R cells, while the horizontal axis indicates the intensity of gene expression in the MIA PaCa-2-A cells. (B) mRNA expression levels of CLDN7 in the MIA PaCa-2-A and MIA PaCa-2-R cells as determined by RT-PCR analysis; *P < 0.01. (C) Western blot analysis of CLDN7 protein expression in the MIA PaCa-2, MIA PaCa-2-A, MIA PaCa-2-R and other human pancreatic cancer cell lines. MCF7 breast cancer cells were used as positive controls for CLDN7 expression.

Table 1
List of genes expressed in the MIA PaCa-2-A cells, but not in the MIA PaCa-2-R cells and whose gene ontology process includes 'adhesion'.

Symbol	Expression in MIA PaCa-2-A cells	Probe_ID	RefSeq_ID	GI	Gene Ontology process
PDZD2	487.1	ILMN_1729095	NM_178140.2	87196342	GO:0007155
TTYH1	426.9	ILMN_1758497	NM_020659.2	53831987	GO:0006811|GO:0006826|GO:0007155
CCL5	29.8	ILMN_1773352	NM_002985.2	22538813	GO:0006874|GO:0006887|GO:0006928|GO:0006935|GO:0006954|GO:0006955|GO:0006968|GO:0006979|GO:0007155|GO:0007165|GO:0007267|GO:0009615|GO:0009615|GO:0045071
BCAM	25.7	ILMN_1790455	NM_005581.3	61742795	GO:0007155|GO:0007155|GO:0007165
RET	24.9	ILMN_1666314	NM_020975.4	126273511	GO:0000165|GO:0001657|GO:0001755|GO:0001838|GO:0006468|GO:0007156|GO:0007165|GO:0007399|GO:0007497|GO:0042551|GO:0048484
CLDN7	20.6	ILMN_2143685	NM_001307.3	34222214	GO:0016338
PKHD1	17.9	ILMN_1749579	NM_170724.2	126131103	GO:0001822|GO:0016337|GO:0042592|GO:0051271
PKP3	17.3	ILMN_1796141	XM_943689.1	89034409	GO:0007155
LAMB4	16.7	ILMN_1792301	NM_007356.2	143770879	GO:0007155
IL32	14.2	ILMN_2368530	NM_001012633.1	61658633	GO:0006952|GO:0006955|GO:0007155

Top 10 genes are listed in order of expression in the MIA PaCa-2-A cells.

[23,24]. The expression of CLDN in human pancreatic cancer tissue has been widely investigated and reported. The expression of CLDN1 has been reported to be associated with epithelial-mesenchymal transition [25] and the TNF-α-dependent cell

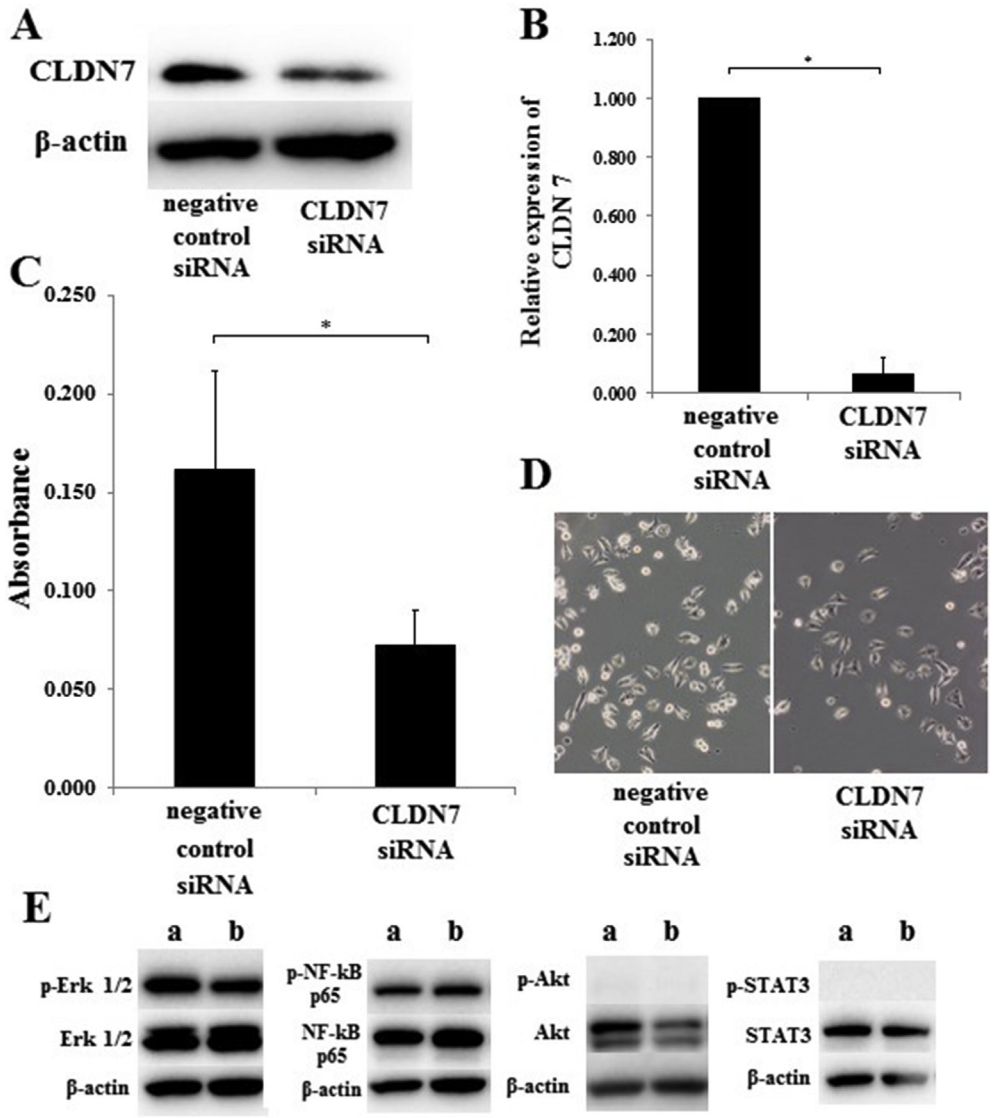

Fig. 4. Knockdown of the CLDN7 gene in the MIA PaCa-2-A cells significantly suppresses cell proliferation without altering cellular morphology. (A) The level of CLDN7 protein in the MIA PaCa-2-A cells transfected with negative control siRNA or CLDN7-specific siRNA for 48 h. CLDN7 protein level was assessed by western blot analysis. (B) The mRNA expression level of CLDN7 in the MIA PaCa-2-A cells transfected with negative control siRNA or CLDN7-specific siRNA for 48 h. The CLDN7 mRNA level was assessed by RT-PCR; $^{*}P < 0.01$. (C) Transfection of the MIA PaCa-2-A cells with CLDN7-specific siRNA for 48 h significantly suppressed cell proliferation. Cell proliferation was assessed using Cell Count Reagent SF; $^{*}p < 0.01$. (D) The morphology of the MIA PaCa-2-A cells was not altered following transfection with CLDN7-specific siRNA. Left panel, MIA PaCa-2-A cells transfected with negative control siRNA for 48 h; right panel, MIA PaCa-2-A cells transfected with CLDN7-specific siRNA for 48 h. (E) Cell signaling of the MIA PaCa-2-A cells transfected with negative control siRNA (lanes labled 'a') or CLDN7-specific siRNA (lanes labeled 'b').

growth [26] of pancreatic cancer. CLDN4 has been reported to be expressed in pancreatic cancer cell lines and human pancreatic cancer tissues [27], and it has been shown to be closely associated with the malignant phenotypes of pancreatic cancer [28]. Although we demonstrated CLDN7 expression in various human pancreatic cancer cell lines, the involvement of CLDN7 in human pancreatic cancer has not yet been defined. Soini et al. [29] reported no association between the expression of CLDN7 and the survival of patients with pancreatic cancer, whereas Alikanoglu et al.[30] demonstrated a significant association of low CLDN7 expression with shorter survival. It is likely that the CLDN family suppresses tumor cell invasion and that a low CLDN expression may promote metastasis, leading to a poor prognosis.

Results from some pre-clinical studies, however, have demonstrated different aspects of CLDN7 as regards the metastasis and proliferation of pancreatic cancer. The knockdown of the CLDN7 gene in highly metastatic pancreatic cancer in rats has been shown to markedly reduce their metastatic ability, possibly as EpCAM-associated CLDN7 inhibits EpCAM-mediated cell-cell adhesion [31]. By cleaving EpCAM, CLDN7 supports the proliferation-promoting activity of EpCAM [32]. In the present study, the proliferation of the MIA PaCa-2-A cells was markedly suppressed by the knockdown of CLDN7. It is conceivable that the EpCAM-promoted cell proliferation may be suppressed by the knockdown of CLDN7 in the MIA PaCa-2-A cells.

The significant accumulation of cells in the G1 phase indicating G1 cell cycle arrest was observed in the MIA PaCa-2-A cells transfected with CLDN7-specific siRNA, and this cell cycle arrest may be, at least in part, the cause of the suppression of the proliferation of the MIA PaCa-2-A cells. The association of the CLDN family with malignant phenotypes, and particularly with cell proliferation and tumor development, is complex, and the controversial

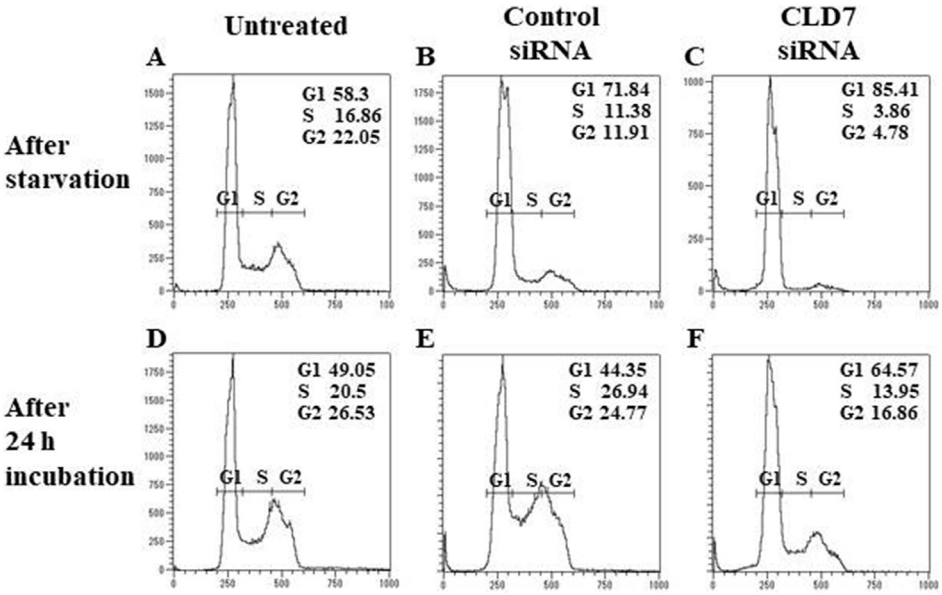

Fig. 5. Knockdown of the CLDN7 gene in the MIA PaCa-2-A cells induced G1 arrest. Cell cycle status of the MIA PaCa-2-A cells untreated (A), transfected with negative control siRNA (B) or CLDN7-specific siRNA (C) immediately after 48 h serum starvation. After serum starvation, cells were subcultured and incubated for 24 h in the complete medium without each siRNA. Cell cycle status of the cells untreated (D), transfected with negative control siRNA (E) or CLDN-7 specific siRNA (F) after 24 h incubation.

involvements of the CLDN family regarding tumor promotion have been reported [41–46]. However, according to the results of the present study, CLDN7 was expressed in the highly malignant MIA PaCa-2-A clones; CLDN 7 expression was low in the MIA PaCa-2-R clones with reduced malignancy. Additionally, the knockdown of CLDN7 significantly suppressed the proliferative activity of the MIA PaCa-2-A cells. Of note, CLDN7 knockdown suppressed the activation of Erk1/2 cell signaling, possibly associated with the suppression of cell proliferation. These results strongly suggest that CLDN7 has potential for use as a novel molecular target for the treatment of pancreatic cancer. Further basic and clinical investigations regarding the nature and clinical association of CLDN7 are warranted in the future.

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Appendix A. Supplementary data

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