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ORIGINAL ARTICLE

CLAUDIN-1 IS ASSOCIATED WITH INVASIVE GROWTH OF HUMAN PANCREATIC CANCER CELLS

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Abstract Claudin is one of tight junction proteins which connect with the actin cytoskeleton and participate in the intracellular signaling. However, the significance of claudin in pancreatic cancer is understood not yet extensively. We examined the relationship between claudin-1 and invasion in PANC-1 and MIA PaCa-2 human pancreatic cancer cells, and investigated the functions of claudin-1 in invasive growth of pancreatic cancer cells. Claudin-1 knockdown by siRNA (claudin siRNA) affected the subcellular localization in the pancreatic cancer cells, and claudin-1 siRNA increased numbers of invasive pancreatic cancer PANC-1 and MIA PaCa-2 cells. Claudin-1 siRNA did not significantly affect expression levels of β-catenin, E-cadherin, α-smooth muscle actin, Bcl-2, and Bax in PANC-1 and MIA PaCa-2 cells. In addition, claudin-1 siRNA showed no significant change in the cell proliferation. We concluded that claudin-1 is significantly associated with invasive growth of human pancreatic cancer cells.

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Key words: claudin; tight junction; pancreatic cancer cells; invasive growth.

原　著

ヒト膵癌細胞における claudin-1 発現と浸潤性増殖の関連

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抄録　細胞接着因子 claudin は，タイトジャンクションを形成し，細胞同士の接着やシグナル伝達などに関連している．しかし，膵癌における claudin の機能は未だ十分に解明されていない．本研究では，ヒト膵癌細胞 PANC-1と MIA PaCa-2 において clauidn-1と浸潤性との関連性を解析した．Claudin-1発現を siRNA により抑制した細胞では，細胞膜に分布する claudin が減少し，マトリゲルを浸潤する細胞数が増加した．また，claudin-1発現を抑制した細胞では，β-catenin, E-cadherin, α-smooth muscle actin, Bcl-2, Bax の発現に変化はなかった．さらに，４型コラーゲン存在下と未処理の場合の細胞増殖能を比較したところ，両者に有意な差は得られなかった．以上より，claudin-1の発現は細胞増殖能に変化を与えることなく，癌細胞の浸潤性増殖に重要な役割を担っていることが示唆された．

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キーワード：クローディン；タイトジャンクション；膵癌細胞；浸潤性増殖．

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Introduction

Tight junctions of cells from epithelial cell to cell adhesion and define cell polarity[1, 2]. They act as a barrier that prevents solutes and water from passing through the paracellular pathway. Tight junction proteins such as claudins, E-cadherin, and β-catenin play important roles in the formation maintenance of the junction. Claudins were first discovered in 1998, and the claudins family consists of at least 24 newly discovered members, the expression of which depends on the cell type and tissue[3-5]. They are connected with the actin cytoskeleton and participate in the intracellular signaling. Alterations of claudin expression have been involved in invasion, metastasis, and formation in several tumor and cancer cells[6, 7]. In previous studies, claudin-1 has regulated cellular transformation and metastatic behavior in colon cancer[6]. Increased expression of claudin-1 has been associated with invasion in oral squamous cell carcinoma[8]. However, relationship between claudins and invasion in pancreatic cancer is understood not yet extensively.

Pancreatic cancer is one of the most lethal malignancies, with a 5-years survival rate of 4%[9-12]. The mean survival time for untreated patients is 3-5 months, while the mean survival after surgical resection ranges from 10-20 months. Factors responsible for this poor prognosis include: a) difficulty in early diagnosis due to anatomical location and lack of early symptoms; b) limitations of conventional cancer therapies including surgery, chemotherapy, radiation therapy, and immune therapy; c) rapid spreading of tumors to the surrounding organs, causing obstructive jaundice; and d) frequent incidence of metastasis even from small primary tumor less than 2 cm in diameter. Pancreatic cancer ranks fifth as a cause of cancer-related mortality in the USA and Japan. Modulation of the aggressive cell proliferation of pancreatic cancer is one of the most significant issues in modern medicine[13, 14].

In this study, we examined modulations of claudin-1 expression in two human pancreatic cancer cell lines, PANC-1 and MIA PaCa-2, and we investigated the relationship between the functions of claudins and invasion of the cancer cells.

Materials and Methods

Cell culture

PANC-1 and MIA PaCa-2 human pancreatic cancer cells (adenocarcinoma) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). PANC-1 cells were cultured in RPMI 1640 medium (GIBCO, Breda, the Netherlands) supplemented with 10% fetal bovine serum. MIA PaCa-2 cells were cultured in Dulbecco's Modified Eagle's Medium-high glucose (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum and 2.5% horse serum. They were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Knockdown of claudin-1/claudin-4 by RNA interference

Short interference RNAs (siRNAs) were used for knockdown of gene expression of claudin-1 or claudin-4. The sequences for the siRNA against claudin-1 (claudin-1 siRNA) were as follows:
sense 5'-r (GCAUGGUAUGGCAAUAGAA) d (TT) -3', and
antisense 5'-r (UUCUAUUGCCAUACCAUGC) d (TG) -3' (claudin-1 siRNA1).
We also used another sequences of claudin-1 siRNA as follows:
sense 5'-r (CGAAAUUGUUACAAUAGAA) d (TT)-3', and
antisense 5'-r (UUCUAUUGUAACAAUUUCG) d (TT)-3' (claudin-1 siRNA2).

The sequences for the siRNA against claudin-4 were as follows:
sense 5'-r (GAGUGGAUGGACGGGUUUA) d (TT) -3', and
antisense 5'-r (UAAACCCGUCCAUCCACUC) d (TG) -3'
The sequences for the negative control siRNA were as follows:
sense 5'-r (UUCUCCGAACGUGUCACGU) d (TT) -3', and
antisense 5'-r (ACGUGACACGUUCGGAGAA) d (TT) -3'.
PANC-1 and MIA PaCa-2 cells were seeded at 5 x 10^4 cells per 35-mm well. 48 h later, the siRNA was transfected into cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). After transfection, cells were incubated for 48 h and subjected to various analyses.

Western Blot analysis

Cells were seeded at 5 x 10^4 cells per 35-mm well and siRNA was transfected into cells using Lipofectamine 2000 reagent. Cells were then lysed using M-PER lysis buffer (PIERCE, Rockford, IL, USA), and proteins were prepared for Western blot analysis. Protein concentrations were determined by BCA (bicinchoninic acid) assay. The lysates (20 μg protein) were subjected to SDS-PAGE, and proteins were transferred to PVDF membranes. The membranes were incubated with antibodies specific for claudin-1 (1:1,000) and claudin-4 (1:5,000) which were purchased from Invitrogen; E-cadherin (1:1000), which was purchased from Takara, Shiga, Japan; β-catenin (1:1000) and Bcl-2 (1:5000), which were purchased from EPITOMICS, CA, USA; Bax (1:5000), which was purchased from Santa Cruz, CA, USA; α-SMA (1:1000) and actin (1:50000) (Sigma) followed by a horseradish peroxidase-conjugated secondary antibody (IBL, Gunma, Japan). Can Get Signal Immunoreaction Enhancer Solution 1 (TOYOBO, Osaka, Japan) was used to dilute the primary antibody. The ECL Plus and ECL Prime Western Blotting Detection System (Amersham, Uppsala, Sweden) was used for detection.

Immunofluorescent staining

The cells were seeded on a 4-chamber slide glass and incubated overnight. The cells were then washed in phosphate-buffered saline (PBS) and fixed with ice-chilled methanol for 30 min, before being permeabilized with 0.2% Triton-X-100 in PBS for 30 min. The permeabilized cells were then washed in PBS twice and treated with 5% normal horse serum in PBS for 30 min (to minimize the non-specific adsorption of antibodies), before being incubated with anti-claudin-1 (1:200) antibodies at 4°C overnight. The cells were then incubated for 1 h with goat anti-rabbit IgG antibody conjugated to Alexa 488 dye (Molecular Probes, Inc, Tokyo, Japan). These cells were visualized using confocal laser scanning microscopy (Zeiss, LSM 710, Wetzlar, Germany).

Matrigel invasion assay

Wells of Matrigel chamber (BD Bioscience, MA, USA) were adapted at room temperature. After claudin-1 or claudin-4 siRNA, PANC-1 and MIA PaCa-2 cells (2.0 x 10^5 cells/ml) were filled with into the upper chambers. Lower chambers were filled with 10% FBS medium to attract cells. Matrigel chambers were incubated for 24 h at 37°C with 5% CO_2. Then, the cells on the upper surfaces of the filter were removed by wiping with a paper. Filter were stained with Giemsa stain solution (WAKO, Osaka, Japan), and the cells on the lower surface of the filter were fixed onto a glass slide. Cells in three randomly selected microscopic fields (x400) of the lower slide were counted. Experiments were performed independently three times.

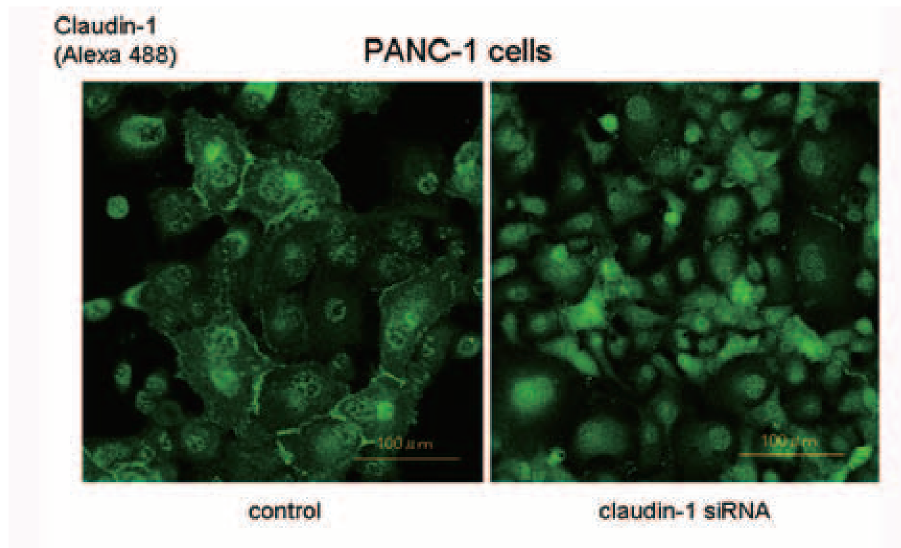

Fig 1 PANC-1 cells were treated with control siRNA or claudin-1 siRNA for 48h, incubated with anti-claudin-1 antibody and visualized using Alexa488. One representative merged image is shown. The cells treated with siRNA claudin-1 were become small and decrease claudin-1 expression in cell membrane.

MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay

Cells were seeded at 5 x 10^4 cells per 35-mm well plates. Cells were transfected with siRNA against claudin-1 and claudin-4. After 2 days, cells were seeded at 1.0 x 10^6 in non coat or collagen-4 coat 96 well plates (BD) for 24h. The cells were added Cell Titer 96 AQ_{uenous} One Solution Reagent (Promega Corporation, Madison, WI, USA) to each well, and the cells were incubated at 37°C for an additional 1 h. The absorbance (OD_{490} nm) was measured using a 96-well plate reader.

Statistics

Data are presented as mean ± SD. Statistical significance was determined by Student's t test. Comparisons with $p < 0.05$ were considered statistically significant.

Results

Subcellular localization of claudin-1 in PANC-1 cells treated with claudin-1 siRNA

We investigated whether the localization of claudin-1 was affected by claudin-1 siRNA-mediated knockdown using immunofluorecent staining. As shown in Figure 1, claudin-1 localized at the cell membranes and nuclei of PANC-1 cells. On the other hand, cells treated with claudin-1 siRNA became shrunken cytoplasm and nuclei in shape, and showed significant decrease of claudin-1 expression at the cell membranes.

Effects of claudin-1/claudin-4 knockdown on cell invasion in PANC-1 and MIA PaCa-2 cells

To examine the roles of claudin-1 and claudin-4 in cell invasion, we performed the siRNA-mediated knockdown of claudin-1 or claudin-4, and detected cell invasion with the Matrigel invasion assay. Claudin-1 siRNA treatment increased numbers of invasive PANC-1 cells through the Matrigel. The invasive PANC-1 cells treated with claudin-1 siRNA showed 80% increase in number compared to cells treated with control siRNA (Figs. 2A, 2B). However, claudin-4 siRNA treatment showed no significant changes of the invasive PANC-1 cells

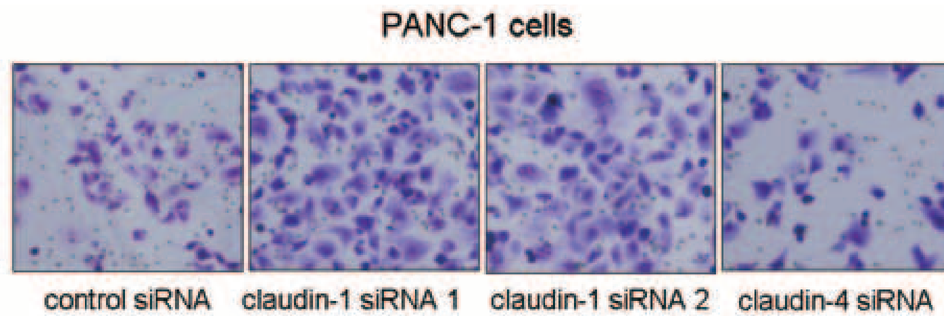

Fig. 2A

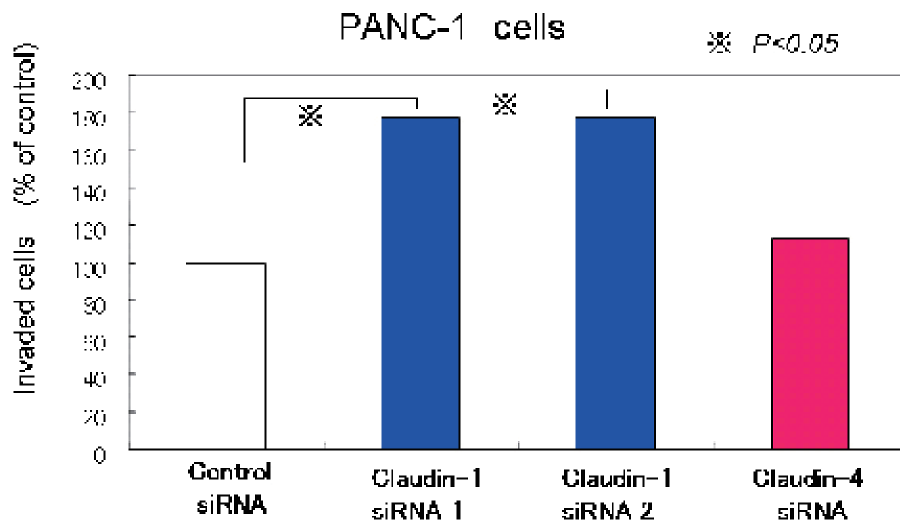

Fig. 2B

in number. On the other hand, claudin-1 siRNA treatment, as well as claudin-4 siRNA treatment, increased numbers of invasive MIA PaCa-2 cells through Matrigel significantly (Figs. 2C, 2D).

Western blotting demonstrated that siRNAs against claudin-1 and claudin-4 suppressed the target genes, i.e. claudin-1 and claudin-4, respectively. Claudin-1 siRNA did not significant affect expression levels of β-catenin, E-cadherin, α-smooth muscle actin (α-SMA), Bcl-2, and Bax in PANC-1 and MIA PaCa-2 cells, while endogenous E-cadherin expression was very week in MIA PaCa-2 cells (Figs. 3A, 3B). Claudin-4 siRNA downregulated β-catenin expression levels, but not expression levels of α-SMA, Bcl-2 and Bax.

Claudin-1/claudin-4 knockdown did not affect cell proliferation in PANC-1 cells

We examined whether the cell proliferation affected by siRNA against claudin-1 or claudin-4 using MTS assay. PANC-1 cells were transfected with MOCK (buffer only), control siRNA, claudin-1 siRNA, or claudin-4 siRNA. After 48 h transfection, the cells were seeded into non-coat or collagen-4 coated 96-well plates at 1 x 10^5 cells/ml, and incubated for 24 h. MTS-assay revealed no significant changes in cell proliferation between the non-coat and collagen-4 coated plates (Fig.4).

Discussion

We focused on the function of claudin-1 in cell

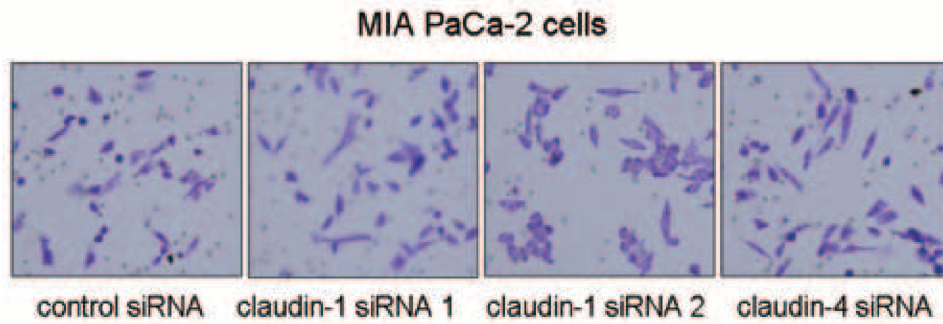

Fig. 2C

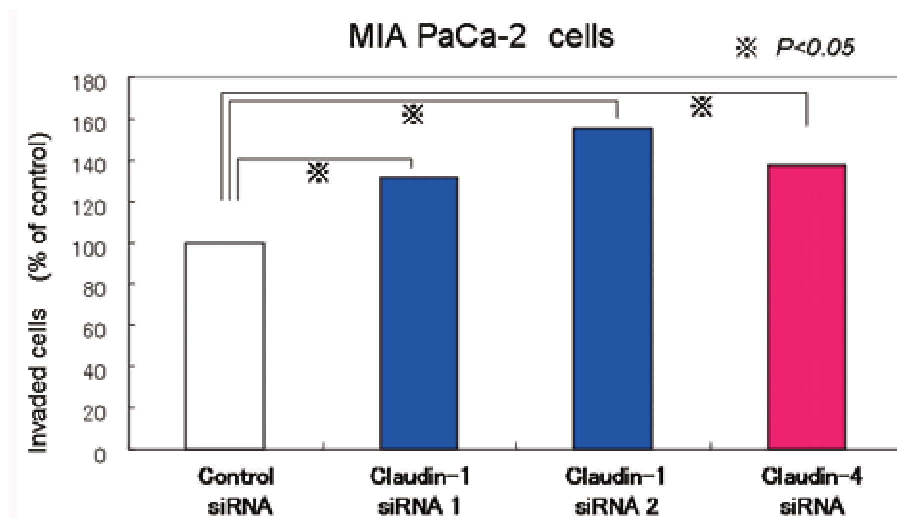

Fig. 2D

Fig 2 PANC-1 and MIA PaCa-2 cells were transfected claudin-1/claudin-4 siRNA and incubated for 48 h. (A) After siRNA, the PANC-1 cells (2.0 x 10^5 cells/ml) were seeded into the upper Matrigel chamber. The invaded cells were stained with Giemsa stain and observed in microscopic fields (x400). (B) Invaded PANC-1 cells were counted in three randomly selected microscopic fields (x400). Cells treated with claudin-1 siRNA showed 80% increase in invasion cells compared to cells treated with control siRNA. Cells treated with claudin-4 siRNA showed no significant changes in invasion cells in cells treated with control siRNA. (C) After siRNA, the MIA PaCa-2 cells (2.0 x 10^5 cells/ml) were seeded into the upper Matrigel chamber. The invaded cells were stained with Giemsa stain and observed in microscopic fields (x400). (D) Invaded MIA PaCa-2 cells were counted in three randomly selected microscopic fields (x400). Cells treated with claudin-1 siRNA showed 30～50% increase in invasion cells compared to cells treated with control siRNA. Cells treated with claudin-4 siRNA showed a 40% increase in invasion cells in cells treated with control siRNA.

invasion of the pancreatic cancer. The claudins are tight junction proteins, that participate in paracellular barrier and cellular connection functions[1-5]. Expression and function of the claudin proteins have not yet been extensively clarified in pancreatic cancer[16-20]. In this study, we demonstrated that claudin-1 knockdown by siRNA affected the subcellular localization in the pancreatic cancer cells, and claudin-1 siRNA increased numbers of invasive pancreatic cancer PANC-1 and MIA PaCa-2 cells. On the other hands, claudin-1 siRNA showed no significant change in the cell proliferation. This is the first study to determine a relationship claudin-1 expression and cell invasion in the human pancreatic cancer cells.

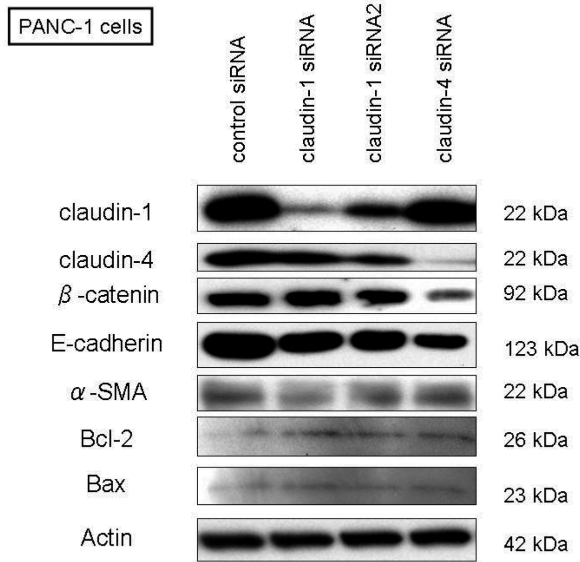

Fig. 3A

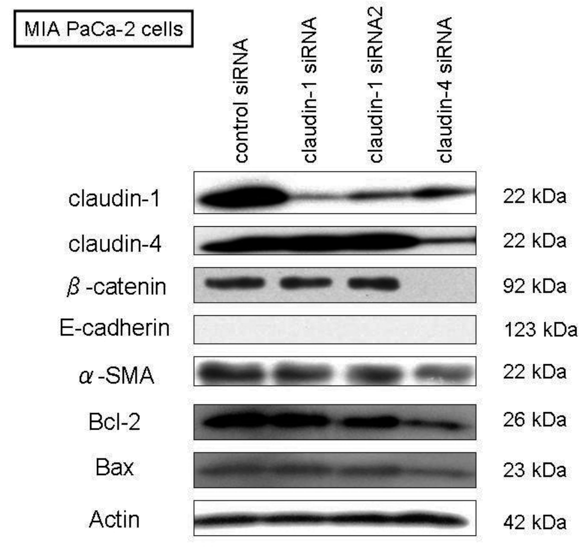

Fig. 3B

Fig 3 Expression of proteins in PANC-1 and MIA PaCa-2 cells transfected siRNA against claudin-1/claudin-4 by western blotting. PANC-1 and MIA PaCa-2 cells were treated with siRNA claudin1/claudin-4 for 48 h.
(A) In PANC-1 cells, there were no significant changes in E-cadherin, α-SMA, Bcl-2 and Bax levels. β-catenin expression was downregulated by claudin-4 siRNA.
(B) In MIA PaCa-2 cells, there were no significant changes in α-SMA, Bcl-2 and Bax levels. β-catenin expression was down-regulated by claudin-4 siRNA. E-cadherin was not detected.

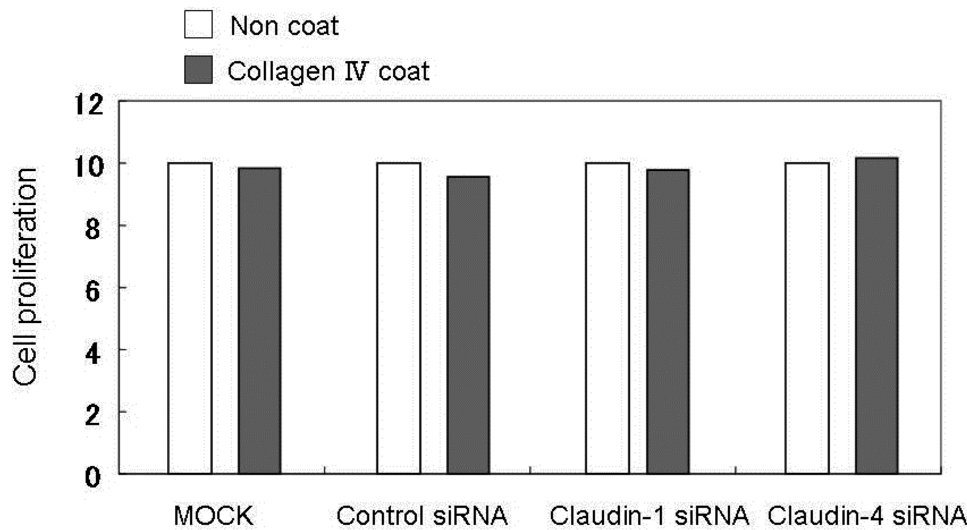

Fig 4 PANC-1 cells were treated with claudin-1/claudin-4 siRNA for 48 h. After siRNA, cells (1.0 x10^6 cells/ml) were seeded in non-coat or collagen-4 coated plates for 24 h. MTS assay demonstrated that collagen-4 did not affect proliferation of claudin-1/claudin-4 siRNA PANC-1 cells.

Recent studies have shown that claudin-1 overexpression increases invasion in oral squamous cell carcinoma, while decreased expression of claudin-1 correlates with recurrence and metastasis in breast cancer[21]. Claudins expression may be related to the regulation of invasion, but these functions were not equal in several cancer cells[22-25]. Knockdown of the β(1) integrin subunit inhibited cell adhesion, migration and

proliferation of pancreatic cancer cells on collagen-4-coated culture[26]. However, the relationship between claudin family and pancreatic cancer cell invasion is clarified not yet extensively. Our study demonstrated that claudin-1 expression was significantly associated with cell invasion, but not cell proliferation. Interestingly, claudin-1 siRNA did not affect expression levels of the other tight junction proteins, such as E-cadherin, while claudin-4 siRNA downregulated β-catenin expression. In addition, claudin-4 was related with cell invasion of only MIA PaCa-2 cells, but not PANC-1 cells. Based on the results, we speculate that claudin-1 play an important role in cell invasion of pancreatic cancer regardless of the other tight junction proteins. On the other hand, claudin-4 is thought to play a role in cell invasion of the specific pancreatic cancer, such as MIA PaCa-2 cells, and may be associated with other tight junction proteins. We also speculated that cell invasion of the pancreatic cancer is independent of cell proliferation. Therefore, we have to suppress cell invasion and proliferation in order to regulate growth of the pancreatic cancer clinically.

Based on the results of the present study, we concluded that claudin-1 is significantly associated with invasive growth of human pancreatic cancer, but not cell proliferation. However, the detailed mechanisms such as molecular interactions have not yet been clarified. In the near future, we will plan to clarify the molecular mechanisms of claudin in cell invasion of human pancreatic cancer.

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References

1) Tsukita S, Furuse M, Itoh M. Multifunctional strands in tight junctions. Nat Rev Mol Cell Biol 2001;2:285-293.

2) Sawada N, Murata M, Kikuchi K, Osanai M, Tobioka H, Kojima T, Chiba H. Tight junctions and human diseases. Med Electron Microsc 2003;36:147-156.

3) Cereijido M, Contreras RG, Shoshani L, Flores-Benitez D, Larre I. Tight junction and polarity interaction in the transporting epithelial phenotype. Biochim Biophys Acta 2008;1778:770-793.

4) Takahashi A, Kondoh M, Yagi K. A non-invasive drug delivery system using claudin binder. Yakugaku Zasshi 2011;131:1583-1587.

5) Borka K. Claudin expression in different pancreatic cancers and its significance in differential diagnostics. Magy Onkol 2009;53:273-278.

6) Takehara M, Nishimura T, Mima S, Mizushima T. Effect of claudin expression on paracellular permeability, migration and invasion of colonic cancer cells. Biol Pharm Bull 2009;32:825-831.

7) Morohashi S, Kusumi T, Sato F, Odagiri H, Chiba H, Yoshihara S, Hakamada K et al. Decreased expression of claudin-1 correlates with recurrence status in breast cancer. Int J Mol Med 2007;20:139-143.

8) Dos Reis PP, Bharadwaj RR, Machado J, Pintilie M, Sukhai MA, Perez-Ordonez B, Gullane P et al. Claudin 1 overexpression increases invasion and is associated with aggressive histological features in oral squamous cell carcinoma. Cancer 2008;113:3169-3180.

9) Fazal S and Saif MW. Supportive and palliative care of pancreatic cancer. JOP 2007;8:240-253.

10) Welsch T, Kleeff J and Friess H. Molecular pathogenesis of pancreatic cancer: advances and challenges. Curr Mol Med 2007;7:504-521.

11) Zavoral M, Minarikova P, Zavada F, Salek C, Minarik M. Molecular biology of pancreatic cancer. World J Gastroenterol 2011;17:2897-2908.

12) Yeh JJ and Der CJ. Targeting signal transduction in pancreatic cancer treatment. Expert Opin Ther Targets 2007;11:673-694.

13) Kijima H, Yamazaki H, Nakamura M, Scanlon KJ, Osamura RY Ueyama Y. Ribozyme against mutant K-ras mRNA suppresses tumor growth of pancreatic cancer. Int J Oncol 2004;24:559-564.

14) Tsuchida T, Kijima H, Hori S, Oshika Y, Tokunaga T, Kawai K, Yamazaki H et al. Adenovirus-mediated anti-K-ras ribozyme induces apoptosis and growth suppression of human pancreatic carcinoma. Cancer gene Ther 2000;7:373-383.

15) Wu YL, Zhang S, Wang GR, Chen YP. Expression transformation of claudin-1 in the process of gastric adenocarcinoma invasion. World J Gastroenterol 2008;14:4943-4948.

16) Lee JW, Hsiao WT, Chen HY, Hsu LP, Chen PR, Lin MD, Chiu SJ et al. Upregulated claudin-1 expression confers resistance to cell death of nasopharyngeal carcinoma cells. Int J Cancer 2010;126:1353-1366.

17) Tsukahara M, Nagai H, Kamiakito T, Kawata H, Takayashiki N, Saito K, Tanaka A. Distinct expression patterns of claudin-1 and claudin-4 in intraductal papillary-mucinous tumors of the pancreas. Pathol Int 2005;55:63-69.

18) Myal Y, Leygue E, Bianchard AA. Claudin 1 in breast tumorigenesis: revelation of a possible novel "claudin high" subset of breast cancers. J biomed Biotechnol 2010;2010:956897.

19) Oliveira SS, Morgado-Diaz JA. Claudins: multi-functional players in epithelial tight junctions and their role in cancer. Cell Mol Life Sci 2007;64:17-28.

20) Dhawan P, Singh AB, Deane NG, No Y, Shiou SR, Schmidt C, Neff J et al. Claudin-1 regulates cellular transformation and metastatic behavior in colon cancer. J Clin Invest 2005;115:1765-1776.

21) Akasaka H, Sato F, Morohashi S, Wu Y, Liu Y, Kondo J et al. Anti-apoptotic effect of claudin-1 in tamoxifen-treated human breast cancer MCF-7 cells. BMC Cancer 2010;10:548.

22) Kondo J, Sato F, Kusumi T, Liu Y, Motonari O, Sato T et al. Claudin-1 expression is induced by tumor necrosis factor-alpha in human pancreatic cancer cells. Int J Mol Med 2008;22:645-649.

23) Hirohashi S and Kanai Y. Cell adhesion system and human cancer morphogenesis. Cancer Sci 2003;94:575-581.

24) Menke A, Philippi C, Vogelmann R, Seidel B, Lutz MP, Adler G et al. Down-regulation of E-cadherin gene expression by collagen type 1 and type 3 in pancreatic cancer cell lines. Cancer Res 2001;61:3508-3517.

25) Shintani Y, Hollingsworth MA, Wheelock MJ, Johnson KR. Collagen 1 promotes metastasis in pancreatic cancer by activating c-jun NH2-terminal kinase 1 and up-regulating N-cadherin expression. Cancer Res 2006;66:11745-11753.

26) Grzesiak JJ, Tran Cao HS, Burton DW, Kaushal S, Vargas F, Clopton P et al. Knockdown of the β(1) integrin subunit reduces primary tumor growth and inhibits pancreatic cancer metastasis. Int J Cancer 2011;129:2905-2915.