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Original Article

Claudin-18 coupled with EGFR/ERK signaling contributes to the malignant potentials of bile duct cancer

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ABSTRACT

Our recent work revealed that elevated expression of claudin-18 is involved in bile duct neoplasia. In the present study, we found that wound generation of a cell sheet *de novo* induced claudin-18 expression in its leading edge, coincident with high mitotic activity. We also found that the suppression of claudin-18 expression significantly reduced cell growth and invasiveness of bile duct cancer cell lines and tumorigenicity *in vivo*. In addition, an antibody specific to an extracellular loop of claudin-18 showed similar effects on the cells such as cell proliferation. Interestingly, treatment with epidermal growth factor (EGF) and overexpression of RAS oncogene induced claudin-18 expression by activation of extracellular signal-related kinase (ERK)1/2. Furthermore, enhanced claudin-18 expression activated ERK1/2. These findings provide evidence for an oncogenic property of claudin-18 in bile duct carcinoma cells via modulation of EGFR/ERK signaling, indicating that claudin-18 is a possible therapeutic target for this malignancy.

Introduction

Biliary tract cancers have an extremely poor outcome because of the difficulty in accurate diagnosis at an early stage of the cancer and limited options for curative treatment consisting only of surgical resections [1]. To improve the prognosis, the establishment of reliable molecular markers is required for accurate diagnosis and surgical resection and for the development of chemotherapy including molecularly targeted therapy. Claudin family proteins have been focused on as useful biomarkers for malignant changes in various tissues [2].

Claudins are tetraspan transmembrane proteins basically localized at tight junctions of cell-cell contact, and the family of claudins consists of 27 members [3,4]. We have recently reported that the expression level of claudin-18 was increased in intraepithelial neoplasia (BilIN) and differentiated bile duct adenocarcinoma and was a useful molecular marker to distinguish the neoplastic region from the non-neoplastic region in both of surgical specimens and biopsies [5]. Shinozaki et al. also reported that claudin-18 expression was increased in intrahepatic and extrahepatic cholangiocarcinomas [6]. Claudin-18 expression was shown to be correlated with poor overall survival and lymph node metastasis of intrahepatic cholangiocarcinoma [6], suggesting that claudin-18 might have a role in biliary carcinogenesis. However, the molecular mechanisms responsible for claudin-18 upregulation in bile duct cancers have not been elucidated. The PKC/AP-1 pathway has been suggested to be involved in the regulation of claudin-18 expression in gastric cancer cells [7] and pancreatic adenocarcinoma cells [8].

In this study, we examined the importance of claudin-18 expression in malignant potentials of bile duct cancer cells and the molecular mechanisms underlying the induction of claudin-18 expression. This is the first report showing that claudin-18 expression is regulated via activation of ERK1/2 and that ectopic claudin-18 expression can activate ERK1/2.

Materials and methods

Antibodies and reagents

The following antibodies were used in this study: rabbit polyclonal anti-claudin-18 (C-terminal, 38-8000), mouse monoclonal anti-occludin (33-1500), Alexa Flour 488 (green)-labeled anti-rabbit IgG, and Alexa Flour 594 (red)-labeled anti-mouse IgG from Thermo Fisher Scientific (Rockford, IL); mouse monoclonal anti-BrdU (clone. Bu20a) and horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG from DAKO (Glostrup, Denmark); anti-ERK1/2 (V1141) from Promega (Madison, WI); mouse monoclonal anti-PKC (pan) (P5704) and rabbit polyclonal

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anti-PCNA (AV03018), rabbit polyclonal anti-actin (A2066) from Sigma-Aldrich (St Louis, MO); rabbit monoclonal anti-phospho-ERK1/2 (Thr202/Tyr204) (#4370), rabbit monoclonal anti-phospho-Akt (Thr308) (#2965), rabbit monoclonal anti-Akt (pan) (#4691) and rabbit polyclonal anti-phospho-PKC (pan, βII Ser660) (#9371) from Cell Signaling Technology (Beverly, MA); and rabbit polyclonal anti-claudin-18 (extracellular loop, AB98071) from Abcam (Cambridge, UK). DAPI, 5-azadeoxycytidine (5-aza-CdR) and TPA were obtained from Sigma-Aldrich. Recombinant human epidermal growth factor (EGF, 100 ng/ml) was obtained from PeproTech EC (London, UK). Inhibitors of ERK (U0126, 10 μM), EGFR (AG1478, 10 μM), PI3K (LY294002, 10 μM), p38 MAPK (SB203580, 10 μM), Pan-PKC (GF109203X, 10 μM), PKCδ (rottlerin, 1 μM), PKCε (PKCε translocation inhibitor peptide, 10 μM), and PKCθ (myristoylated PKCθ pseudosubstrate peptide inhibitor, 10 μM), and PKCα (Gö6976, 1 μM) were obtained from Merk (Darmstadt, Germany).

Cell cultures and treatment

Human bile duct adenocarcinoma cell lines TKKK, OZ and HuCCT1 were purchased from ATCC (Manassas, VA) and maintained in Dulbecco's Modified Eagle Medium (DMEM) (TKKK and OZ) or RPMI-1640 (HuCCT1) (Sigma-Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Rockford, IL). The medium for the cell lines contained 100 U/ml penicillin and 100 μg/ml streptomycin, and all of the cells were plated on 35- or 60-mm culture dishes (Corning Glass Works, Corning, NY) that had been coated with rat tail collagen (500 μg of dried tendon/ml in 0.9% acetic acid) and incubated in a humidified 5% CO_2 incubator at 37 °C.

Transfection

cDNA of full-length human claudin-18 variant 2 (v2) was obtained by RT-PCR from total RNA of TKKK cells and was cloned into pcDNA3.1(+) (Thermo Fisher Scientific). pBABEpuro-RAS V12, a vector expressing constitutively active H-RAS (G12V) mutant, was purchased from Cell Biolabs (San Diego, CA). Small interference RNAs (siRNAs) duplex targeting the mRNA sequences of human claudin-18 (v2) and scrambled Stealth RNAi were purchased from Thermo Fisher Scientific. Sequences are CCCUGAUGAUCGUAGGCAUCGUCCU and AGGACGAUGCCUACGAUCAUCAGGG (CLDN18-A), GGGUCGCUGGAGGCCUCACACUAAU and AUUAGUGUGAGGCCUCCAGCGACCC (CLDN18-B), GCACAGAGGACGAGGUACAAUCUUA and UAAGAUUGUACCUCGUCCUCUGUG (CLDN18-C). Transfection was performed as described previously [9].

Reverse transcription (RT)-PCR and western blotting

RT-PCR and Western blot analysis were performed as described previously [8,9]. Immunoreactive bands of western blots were detected by the Clarity™ western ECL Substrate system (Bio-Rad Laboratories, Hercules, CA) using X-ray film (Kodak, Rochester, NY) or AE-9300H Ez-Capture MG (ATTO, Tokyo, Japan).

Cell proliferation and invasion assays

Incorporation of 5-bromo-2-deoxyuridine (BrdU) (Sigma-Aldrich, St Louis, MO) into cellular DNA was used to determine proliferation rate. Cells were grown on 35-mm glass-base dishes (Iwaki, Chiba, Japan) coated with rat tail collagen. The cells were incubated for 2 h after treatment with 20 μM BrdU and were then fixed in cold absolute ethanol. After the samples had been incubated with 2 N HCl at room temperature for 20 min, they were incubated with rabbit anti-claudin-18 antibody (C-term, 1:100) and mouse anti-BrdU antibody (1:200) at room temperature for 1 h and then with Alexa 488 (green)-conjugated anti-rabbit IgG antibody (1:200) and Alexa 594 (red)-conjugated anti-mouse IgG antibody (1:400) at room temperature for 1 h. DAPI was used for counterstaining of nuclei in the cells. The number of cells with BrdU-labeled nuclei was counted using an epifluorescence microscope (Olympus, Tokyo, Japan). More than 1000 cells were counted per dish, and three dishes were examined per experiment. To examine the inhibitory effect of the antibody, cells were incubated in a medium containing 1 or 10 μg/ml of the antibody for 24 h and rinsed with PBS twice, and then proliferation rate was examined by the BrdU incorporation assay. In the WST-1 assay, cells were seeded in 96-well plates. After 24 h of incubation, cell viability was assessed by using a cell counting kit-1 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Absorbance at a wavelength of 450 nm was measured using a Bio-Rad Model 680 microplate reader (Bio-Rad). For the invasion assay, we used a Corning BioCoat Matrigel Invasion Chamber (Thermo Fisher Scientific) according to the manufacturer's instructions. Cells in a medium without FBS were plated onto the Matrigel chambers at 48 h after transfection. The lower chamber of the Transwell was filled with a medium containing 10% FBS. At 24 h after plating, invading cells were fixed and visualized by 0.04% Crystal violet and 2% ethanol for 10 min. The areas of invading cells were measured using a microscope imaging system (Olympus).

Xenograft model

Studies were performed in 8-week-old BALB/cAJcl-nu/nu female mice (HOKUDO, Japan). At 48 h after siRNA transfection, 1×10^6 TKKK cells were collected in 100 μl of PBS and then mixed with 100 μl of Matrigel. The cell/Matrigel mixture was subcutaneously injected into the hind legs of anesthetized mice. Tumor volume was measured weekly. All animal experiments conformed to the National Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Committee at Sapporo Medical University.

Statistical analysis

The measured values are presented as means ± SD. Statistical significance of differences was evaluated using the unpaired Student's t-test.

Results

Claudin-18 is overexpressed at the growing edges of bile duct adenocarcinoma cell sheets

All of the bile duct adenocarcinoma cell lines used in this study expressed constitutive claudin-18 at various levels (Supplementary Fig. S1A). 5-Azadeoxycytidine (5-aza-CdR), an inhibitor of DNA methylation, increased claudin-18 expression in HuCCT1 cells (Supplementary Figs. S1B and S1C), suggesting that the regulatory sequence of claudin-18 is probably methylated in the cell line.

Interestingly, TKKK cells showed heterogeneous staining with anti-claudin-18 antibody, different to the homogeneous staining pattern of occludin (Fig. 1A). Strong immunoreactivity was observed along the edges of cell sheets. BrdU labeling with double-immunostaining of anti-claudin-18 and anti-BrdU antibodies showed that the claudin-18-positive rate was significantly higher in BrdU-positive cells than in BrdU-negative cells (Fig. 1B, $p < 0.05$), suggesting that claudin-18 expression was upregulated in proliferating cells.

Claudin-18 contributes to proliferation, invasion and in vivo tumorigenesis of bile duct adenocarcinoma cells

To determine whether claudin-18 expression has a role in malignant potential, we knocked down claudin-18 expression and evaluated its effect. We employed three different claudin-18-specific siRNAs, and all of them effectively reduced claudin-18 expression both in mRNA and protein levels in TKKK cells (Fig. 2A and Supplementary Fig. S1D). In BrdU labeling analysis, the percentage of BrdU-positive cells was significantly lower in claudin-18 knockdown (KD) cells than in control cells (Fig. 2B and C, $p < 0.001$). In the WST-1 cell proliferation assay, the percentage of proliferative cells in claudin-18 KD cells was significantly lower than that in control cells at 120 h after siRNA transfection (Fig. 2D, $p < 0.05$). These results indicate that the expression level of claudin-18 modulates cell proliferation.

We also found that an extracellular domain of claudin-18, the second extracellular loop (ECL) of the tetra-span-transmembrane protein, was involved in cell proliferation. Cell proliferation was significantly inhibited when cells were pretreated with an anti-claudin-18 antibody specific to the second ECL at the low dose of 1 μg/ml ($p < 0.001$ for TKKK cells in Fig. 2F and G; $p < 0.001$ for OZ cells in Supplementary Fig. S2). In contrast, an anti-claudin-18 antibody specific to the intracellular carboxyterminal (C-term) region had no inhibitory effect on cell proliferation even at a higher dose of 10 μg/ml (Fig. 2F and G and Supplementary Fig. S2).

In an invasion assay using a Matrigel matrix-coated chamber, the number of claudin-18 KD cells penetrating through the matrix was significantly smaller than that of control cells (Fig. 2E, $p < 0.001$). In a mouse xenograft model, tumors derived from claudin-18 KD cells developed more slowly than tumors from control cells (Fig. 3A). Resected tumor weight at day 56 was significantly smaller for tumors from claudin-18 KD cells than for tumors from control cells (Fig. 3B, $p < 0.001$). These results indicated that claudin-18 contributes to cell proliferation, invasion and *in vivo* tumorigenesis.