RESEARCH ARTICLE

Overexpression of Claudin-4 in Cholangiocarcinoma Tissues and its Possible Role in Tumor Metastasis

Suphawadee Bunthot[1,3], Sumalee Obchoei[1,3], Rattaphol Kraiklang[1,3], Chawalit Pirojkul[2,3], Sopit Wongkham[1,3], Chaisiri Wongkham[1,3]*

Abstract

Claudin-4 (CLDN4) is a tight junction protein that forms apical junctional complexes in epithelial and endothelial cellular sheets. Acting as a barrier and control of permeability are the general functions of tight junction proteins contributing to tissue homeostasis, paracellular ion flux, and cell-cell contact. In this study, we immunohistochemically examined CLDN4 expression in liver fluke-associated cholangiocarcinomas (CCAs) with tissue microarrays. Regardless of the histological type and gross type of cancer, high expression of CLDN4 was noted in precancerous hyperplastic/dysplastic biliary epithelia and CCA. To investigate functional roles of CLDN4 in cancer progression, the effects of CLDN4 suppression by siRNA on cell proliferation, migration and invasion were investigated in two CCA cell lines, KKU-M139 and KKU-M213. Suppression of CLDN4 expression did not alter cell proliferation but caused significant reduction of cell migration and invasion by both CCA cell lines. Our results suggest that over-expressed CLDN4 may promote CCA expansion and metastasis.

Keywords: Bile duct cancer - CLDN4 - proliferation - migration - invasion

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Introduction

Cholangiocarcinoma (CCA) is a lethal cancer with poor prognosis. CCA is a rare cancer, however, the incidence rates has been rising worldwide in the past decades (Landis et al., 1998; Patel, 2002). The incidence of CCA varies in the different geographic regions and it is highest in the northeastern Thailand (Bragazzi et al., 2012). There are several factors identified as risks for CCA. Primary sclerosing cholangitis, hepatolithiasis, and choledochal cysts are the risk factors for CCA in the western countries, whereas liver fluke infection (*Opisthorchis viverrini*) is proven to be the strong risk for CCA in Thailand. Still, the molecular mechanisms of carcinogenesis and progression of CCA are unclear.

Claudins (CLDNs), a family of transmembrane proteins, are integral components of tight junctions that play important roles in maintaining epithelial cell polarity, controlling paracellular diffusion, and regulating cell growth and differentiation (Matter et al., 2005). The distribution and expression levels of CLDNs have been found to vary in different organs and tissues. Up to now, there are 24 types of CLDNs found so far. Of these, CLDN4 is the most attractive in cancer research as *Clostridium perfringens* enterotoxin can bind specifically to CLDN4, and induces cell apoptosis *in vitro* (Chakrabarti and McClane, 2005), and suppressed tumor growth in an animal model (Michl et al., 2001; Kominsky et al., 2007), suggesting a possible future therapeutic target. Roles of CLDN4 in carcinogenesis and progression in cancers have been suggested. CLDN4 is expressed differently in various fetal and adult tissues. CLDN4 expression is frequently elevated in various cancers such as colorectal, ovarian, gastric, breast, prostatic, and pancreatic cancers (Michl et al., 2003; Rangel et al., 2003; Soini, 2005).

CLDN4 is highly expressed in both intrahepatic and extraheptic CCA (Lodi et al., 2006). Higher expression of CLDN4 in CCA than hepatoma was indicated by immunohistochemistry (Lodi et al., 2006) and serial analysis of gene expression (Nishino et al., 2008) and hence CLDN4 was suggested to be the marker to differentiate CCA from hepatoma. Recently, CLDN2, 3, 4 and 10 expression in intrachepatic and extrahepatic bile duct and gall bladder cancers were studied (Nemeth et al., 2009). A strong expression of CLDN4 was found in extrachepatic CCA and gall bladder carcinoma, whereas CLDN4 in intrahepatic CCA was weakly expressed and not different of that in the normal bile duct epithelia. All those previous reports of CLDN4 in CCA clinical specimens were from non-liver fluke associated CCA patients and the level of CLDN4 expression seems to be controversial in those reports. In the present study, we examined the expression profile of CLDN4 in clinical specimens from the liver fluke associated CCA patients. In addition, the roles of CLDN4 on cell proliferation, migration, and invasion in CCA cell lines were examined.

*[1]Department of Biochemistry, [2]Department of Pathology, [3]Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Thailand *For correspondence: chaisiri@kku.ac.th*

Materials and Methods

Tissue and immunohistochemistry

The paraffin embedded tissue-microarrays were prepared from 118 cases of histologically proven CCA tissues collected in the specimen bank of Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University. The protocol for collection and study were approved by the Ethics Committee for Human Research, Khon Kaen University (HE5215209). Briefly, the tissues were deparaffinized and incubated with 1:50 of primary antibody against CLDN4 (Santa Cruz Biotechnology, CA) at room temperature overnight, washed, and incubated with a rabbit anti-goat conjugated peroxidase (Histofine, Tokyo, Japan) at room temperature for 2 h. The peroxidase activity was observed using diaminobenzidine tetrahydroxychloride solution (DAB; Dako; Glostrup, Denmark) as the substrate. Mayer's hematoxylin was used as nuclear counterstain. The brown-membranous staining was counted as CLDN4 positive. Since all samples were positive with CLDN4-immunoreactivity, only the intensity of the staining was scored as follows: negative staining=0; weak staining=+1; moderately staining=+2; and strong staining=+3. For univariate analysis, the immunohistochemistry scores 0 to +1 were considered low, and scores 2 and 3 were considered as high CLDN4 expression.

Cell lines

Six human CCA cell lines, KKU-M055, KKU-100, KKU-M139, KKU-M156, KKU-M213 and KKU-M214, were established from different histological types of primary tumors from CCA patients (Sripa et al., 2005). Cell lines KKU-M055 and KKU-100 originated from patients with poorly differentiated adenocarcinoma. KKU-M156 and KKU-M214 were derived from moderately differentiated CCA, while KKU-M139 and KKU-M213 were established from squamous carcinoma and adenosquamous CCA, respectively. An immortalized human cholangiocyte cell line, MMNK1, was established as described previously (Maruyama et al., 2004) and used in this study as a non CCA cell line for comparison. All cell lines were cultured in DMEM (Invitrogen, NY), containing 100 U/mL penicillin and 100 µg/mL streptomycin supplemented with 10% fetal bovine serum at 37°C with 5% CO_2.

Small interfering RNA knockdown experiments

KKU-M139 and KKU-M213 cell lines were cultured in a 6-well plate and transfected with siRNA-CLDN4 duplexes (Invitrogen) using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Nonspecific siRNA duplexes (scramble control) were used as controls. The efficiency of siRNA was monitored from 24 h-96 h using Western blotting.

Real time RT-PCR

Relative real time RT-PCR was performed using LightCycler 480 II (Roche Diagnostics, Rotkreuz, Switzerland) with 1X LightCycler® 480 SYBR Green I Master (Roche Applied Science, Mannheim, Germany). CLDN4 was performed with primers: F5'-TACATTTTCCCCACTCTGTC-3' and R5'-CAAACCTGTTTACAGCACCT-3'. β-2-Microglobulin was used as an internal control with primers: F5'-AAGATGAGTATGCCTGCCG-3' and R5'-CGGCATCTTCAAACCTCC-3'. The reaction mixtures contained 20 ng of cDNA, 1X LightCycler® 480 SYBR Green I Master and, 400 nM of primers at the final volume of 10 μL. The assays were performed in duplicate, in an optical 96-well reaction plate and template negative reactions were used as negative controls. The thermocycling program was 50 cycles of 95ºC for 10 sec, 55ºC for 10 sec, and 72ºC for 3 sec with an initial cycle of 95ºC for 5 min. At each cycle, accumulation of PCR was detected by monitoring the increase of fluorescence of the dsDNA-binding LightCycler® 480 SYBR Green I Master. After the PCR, a dissociation curve was constructed in the range of 60-99ºC. All data were analyzed using the LightCycler® 480 SW 1.5 software.

Western blotting

Cells were lyzed with lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, and 1% NP-40) for 15 min on ice. Cell lysates were then collected after centrifugation at 14,000 rpm for 20 min at 4°C. Total protein (15 μg) was separated by a 4-20% Tris-Glycine gradient SDS polyacrylamide gel electrophoresis which was transferred onto a Hybond-P PVDF membrane (GE healthcare, Buckinghamshire, UK) for 2 h at 4°C. The membrane was blocked with 3% bovine serum albumin, washed in 0.3% Tween 20-Tris buffered saline, and probed with 1:500 of goat anti-CLDN4 antibody, (Santa Cruz Biotechnology) at room temperature for 1 h. The blot was then washed and incubated in 1:10,000 of rabbit anti-goat-horseradish peroxidase-conjugated secondary antibody (Dako Cytometion, Glostrup, Denmark) for 1 h at room temperature and then washed. The immunoactive bands were detected using an ECL kit (GE healthcare).

Cell proliferation

The effects of CLDN4 knockdown on cell proliferation were determined by measuring cell viability using the 3-[4,5-dimethylthiazole]-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the cells were seeded into 96-well plates (3×10^3 cells/well) and serum-starved (0% FBS) for 6 h. Then, cells were incubated with the DMEM medium supplemented with 5% FBS. Cell growth was assessed at 0, 24, 48, and 72 h. The MTT reagent (10 μL of MTT mixed with 100 µL of phosphate buffer saline, pH7.4) was added to each well and incubated at 37°C for 4 h. Then, 100 μL of 0.04N HCl-isopropanol was added and the absorbance was recorded at 570 nm.

Invasion and cell migration assay

Cell invasion capability was determined using Boyden chamber assay. Transwell inserts of an 8 μm pore size (Corninge, NY) were coated with 100 μL of 40 mg/mL of Matrigel (BD Biosciences, MA) in DMEM. Cells (4×10^4 cells/well) in 100 μL of serum-free DMEM were plated onto a pre-coated insert and DMEM containing 10% FBS (600 μL) was added in the lower chamber. The

assay plate was incubated at 37°C, 9 h for KKU-M213 and 14 h for KKU-M139. The invaded cells were fixed in 4% paraformaldehyde and stained with 0.4% (w/v) sulforhodamine B sodium salt in 1% acetic acid and counted under a microscope (20x magnification). The cell migration assay was carried out essentially as mentioned above, except that the cells were placed on top of an uncoated insert. At least three independent experiments were done for the assay.

Statistic analysis

Data were analyzed using SPSS 16.0 software (SPSS, Chicago, IL). The associations between the immunohistochemistry scores and clinicopathological features of patients were analyzed using Chi square. The differences between the mean values were determined using Students't test. Survival analysis was performed using Kaplan-Meier plot and Log-Rank test. P-values of <0.05 were considered significant.

Results

CLDN4 is overexpressed in cholangiocarcinoma tissues

Claudin 4 was detected in a tissue-microarray section containing 118 histological CCA proven tissues. To ascertain the expression of CLDN4 in normal bile duct epithelia, the normal bile ducts in the liver tissues from patients with hemangioma, and hepatocellular carcinoma were used in addition to those observed in the adjacent non-tumorous area of CCA tissues. Unrelated to the type of tissue origin, all normal bile duct epithelia were weakly positive for CLDN4 (Figure 1A). The hyperplastic and dysplastic bile ducts exhibited strong expression of CLDN4 (Figure 1B). All CCA tissues were positive for CLDN4 with different intensities. Diffuse and membranous staining patterns with high intensity of CLDN4 immunoreactivity was shown in 61% (72/118) of CCA regardless to histotype. High expression of CLDN4 was found in CCA with papillary (Figure 1C) and non-papillary types (Figure 1D). The expression level of CLDN4 was analyzed in association with the clinicopathological parameters of CCA patients. We did not find significant correlations between CLDN4 and clinicophatological parameters (Table 1) or overall survival of the patients (Figure 2).

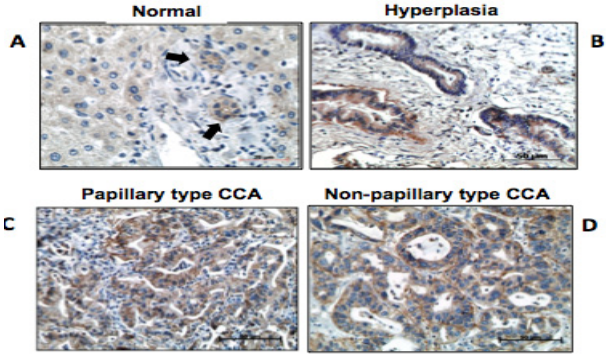

Figure 1. CLDN4 Expression in Normal and CCA Bile Duct Epithelia. CLDN4 expression in CCA tissue-microarrays were determined. The immunoreactivity of CLDN4 was weakly observed in (A) normal bile duct epithelia (arrows) and hepatocytes (400x magnification); but was strongly observed in (B) hyperplastic/dysplastic bile duct; (C) papillary and (D) non-papillary types of CCA. B-D, 200x magnification

Endogenous expression of CLDN4 in cholangiocarcinoma cell lines

We first examined the CLDN4 expression in six CCA cell lines compared to that of an immortalized human cholangiocyte cell line. As shown in Figure 3, all CCA cell lines expressed CLDN4 at different levels as shown by mRNA (Figure 3A) and Western blotting (Figure 3B). KKU-M139, KKU-M156, KKU-M213 and KKU-M214 highly expressed CLDN4, whereas MMNK1, KKU-M055 and KKU-100 had low expression. The CLDN4 proteins of each cell lines corresponded well with the mRNA levels.

Table 1. Univariate Analysis for Correlation between CLDN4 Expression and Clinical Features of CCA Patients

Variables		N	CLDN4		p value
			Low	High	
Age (years)	<56	56	20	36	0.308
	>56	62	26	36	
Sex (M:F=1:1.8)	Male	76	31	45	0.367
	Female	42	15	27	
Histological type	Papillary	47	18	29	0.528
	Non-papillary	71	28	43	
Gross type	Mass forming	79	29	50	0.458
	Periductal infiltrating	29	14	15	
	Intraductal growth	10	3	7	
Lymphatic invasion	Yes	52	21	31	0.508
	No	24	9	15	

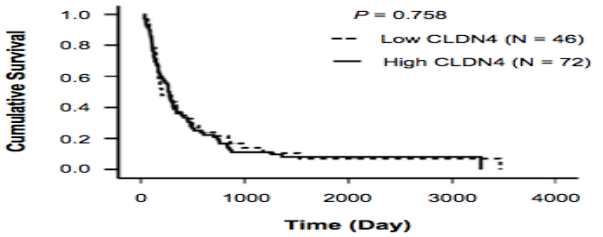

Figure 2. Correlation between CLDN4 Expression and Cumulative Survival Rate (Kaplan-Meier Method). The survival of patients with low CLDN4 expression was not different from those with high CLDN4 expression (Log rank; P=0.758)

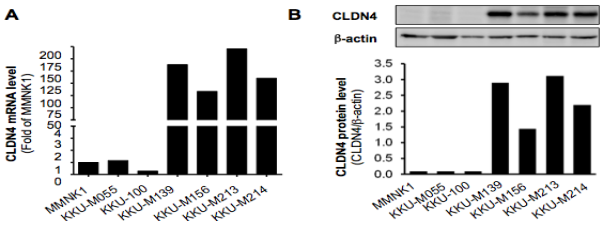

Figure 3. Expression Levels of CLDN4 in CCA and Immortalized Cholangiocyte Cell Lines. Using the expression level of CLDN4 of immortalized cholangiocyte cell lines (MMNK1) as controls, the expression of CLDN4 in 6 CCA cell lines was determined and compared to: (A) mRNA expression levels using real time RT-PCR and (B) protein levels using Western blot analysis and image-quantitative analysis. The levels of CLDN4-mRNA corresponded well with those of CLDN4 proteins

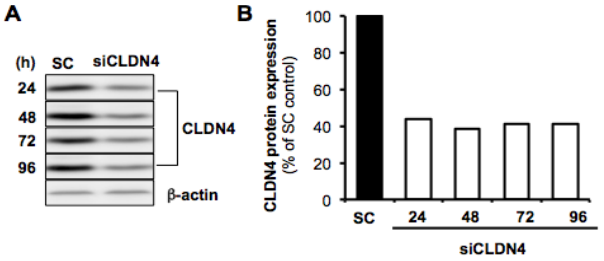

Figure 4. siCLDN4 Significantly Suppressed CLDN4 Expression of CCA Cell Line. The expression of CLDN4 in KKU-M213 cells was efficiently suppressed by siRNA against CLDN4 (siCLDN4) up to 96 h. Nonspecific siRNA (scramble, SC) were used as negative controls and β-actin was used as an internal control. (A) Western blotting, (B) the intensity of each band was quantitatively analyzed as percent of scramble control of each time point

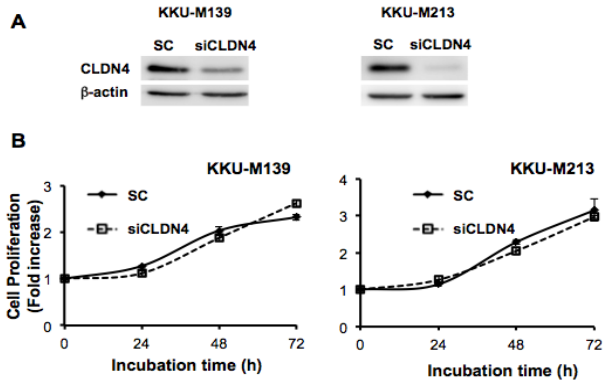

Figure 5. Effect of CLDN4 on Proliferation of CCA Cell Lines. CLDN4 expression in CCA cells were suppressed using siCLDN4. siCLDN4 treated cells were subjected to proliferation assays for 72 h. (A) Western blotting of CLDN4 indicated that CLDN4 expressions in the siCLDN4 treated cells were significantly lower than those of scramble treated control cells (sc) in both KKU-M139 and KKU-M213 cell lines. (B) Proliferation rates of siCLDN4 treated KKU-M139 and KKU-M213 cells (dotted lines) were not different from those of the controls (solid lines)

CCA cell lines, KKU-M139 and M213, were selected for siCLDN4 suppression in the subsequent study.

Suppression of CLDN4 expression does not affect CCA cell proliferation

Transient suppressions of CLDN4 expression in KKU-M139 and KKU-M213 cell lines were conducted using siRNA specifically to CLDN4. Cells treated with scramble RNA were used as controls. The efficiency of siCLDN4 was checked in KKU-M213 cells at 24 to 96 h after transfection. The Western blot analysis of CLDN4 indicated that siCLDN4 could suppress CLDN4 expression to ~40% of the controls up to 96 h (Figure 4). Proliferation rates of the siCLDN4 treated KKU-M139 and KKU-M213 cells compared to the scramble controls were observed for 72 h. Suppression of CLDN4 expression did not affect the proliferation rates of both KKU-M139 and KKU-M213 cells compared to the controls (Figure 5).

Suppression of CLDN4 inhibits migration and invasion of CCA

Migration and invasion activities of CCA cells

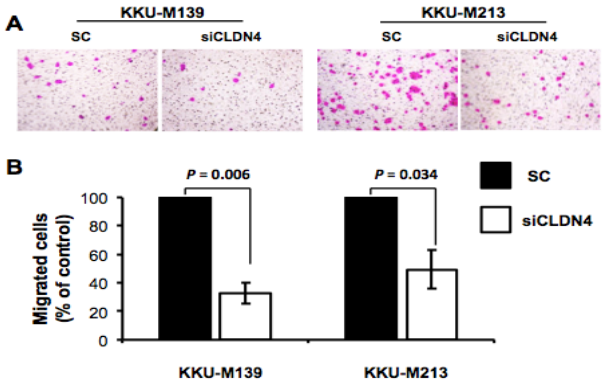

Figure 6. Effect of CLDN4 on Migration Activity of CCA Cell Lines. The effect of CLDN4 on migration activity was investigated using the Boyden chamber assay. Suppression of CLDN4 expression by siCLDN4 significantly reduced the number of migrated cells compared to the scramble controls (sc). (A) The representative of migrated cells; (B) Number of migrated cells of siCLDN4 treated CCA cells from the KKU-M139 and KKU-M213 cell lines were significantly lower than the controls. The data are one representation of three separate experiments

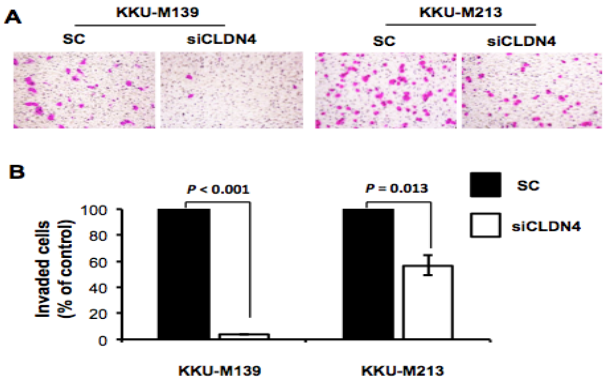

Figure 7. Effect of CLDN4 on Invasion Activity in CCA Cell Lines. The effect of CLDN4 on invasion activity was investigated using the Boyden chamber assay. Suppression of CLDN4 expression by siCLDN4 significantly reduced the number of invaded cells compared to the scramble control (sc) treated cells. (A) Representation of invaded cells; (B) Number of invaded cells of siCLDN4 treated CCA cells, KKU-M139 and KKU-M213 lines were significantly lower than the controls. These data are one representation of three separate experiments

were evaluated using the Boyden chamber assay. As compared to the scrambled controls, the migration of siCLDN4 treated cells was significantly reduced to 30% in KKU-M139 (P=0.006) and to 50% in KKU-M213 (P=0.034) cells (Figure 6). A similar observation was obtained in the invasion assay. Suppression of CLDN4 significantly decreased number of invaded cells to less than 10% in siCLDN4 treated KKU-M139 (P<0.001) and to <60% in KKU-M213 (P=0.013) cells (Figure 7).

Discussion

CLDN4 expression is frequently altered in various types of cancers. Many studies have shown the elevation of CLDN4 in association with cancer progression (Li et al., 2009; Hwang et al., 2010). In this study, we have demonstrated for the first time that CLDN4 was over-expressed in the hyperplastic/dysplastic and malignant bile duct epithelia of the liver fluke associated CCA.

Comparing to the normal bile duct, CLDN4 was highly expressed in CCA regardless of the tissue type and site of tumor origins. The involvement of CLDN4 in tumor migration and invasion but not proliferation of CCA cell lines was clearly shown in this study.

We examined the expression of CLDN4 in 118 histological proven-intrahepatic CCA tissues. Weak expression of CLDN4 in normal bile duct epithelia of the non-tumorous liver tissues from CCA patients was confirmed by determining CLDN4 in normal bile duct epithelia of liver tissues from various liver pathologic patients, namely hemangioma and hepatoma, and normal cadaveric donors. All normal bile duct epithelia showed weak CLDN4 expression. In contrast, the precancerous hyperplastic/dysplastic and all malignant bile duct epithelia of intrahepatic CCA over-expressed CLDN4. Strong expression of CLDN4 was also observed in 5/7 cases of extra-hepatic CCA (data not shown). These observations indicate the important role of CLDN4 in tumorigenesis of CCA. Our results from Thai patients who are accepted to be the liver fluke-associated CCA patients supported those of Lodi, et al. (2006) who reported CCA in Hungarians and Nishino, et al. (2008) observations in Japanese CCA.

The definite roles of CLDN4 in tumorigenesis and tumor progression have remained unclear. However, there are several studies that have shown the possible functions of CLDN4 in promoting cell migration and invasion of cancer cells from pancreas (Michl et al., 2003), colon (Takehara et al., 2009), and ovary (Agarwal et al., 2005). The loss of cell-cell contact and cell-extracellular matrix could promote cell migration and invasion. Suppression of CLDN4 may reduce these contacts and encourage migration and invasion activities of cells. In our study, siCLDN4 effectively suppressed CLDN4 expression in CCA cell lines, KKU-M139 and KKU-M213 without disturbing of proliferation rates. However, suppression of CLDN4 expression did significantly reduce cell migration and invasion of both cell lines. Among 6 CCA cell lines tested in this study, endogenous expression of CLDN4 of KKU-100 and KKU-M055 was much lower than that of KKU-M139, KKU-M156, KKU-M213, and KKU-M214. The association of migration and invasion abilities of CCA cells with the expression level of CLDN4 was found in the study reported by Junking et al (2008). The numbers of invaded cells of KKU-M214 (high expression of CLDN4) were higher than those of KKU-100 (low expression of CLDN4).

Increased matrix metalloproteinase-2 (MMP-2) and MMP-9 activities in claudin-expressing tumor cells were seen in gastric, ovarian, and colon cancers, indicating that claudin-mediated increased invasion might be mediated through the activation of MMP proteins (Agarwal et al., 2005; Takehara et al., 2009; Hwang et al., 2010). The association of CLDN4 in promoting angiogenesis was also observed in ovarian cancer. It was shown that ovarian cancer cells which expressed claudin-4 secreted factors that can mediate angiogenesis in the dorsal skin of mice (Li et al., 2009).

Claudin proteins are crucial for tight junction formation and function. The epical junction complex is not static, but an extremely dynamic structure. The functions of tight junctions are identified in several biological events, e.g., during epithelial tissue remodeling (Peralta et al., 1996), wound repair (Mooradian et al., 1992), inflammation (Riehl and Stenson 1994), and transformation into tumors (Madara et al., 1992). The mechanisms that regulate these phenomena are poorly understood. Although literature regarding CLDNs identified CLDNs as a main structure of tight junction, however, interactions of CLDNs with several cellular signaling molecules are repeatedly reported. Carboxyl terminus of CLDNs was supposed to be involved in signaling pathways via the binding domain to zonula occludens (ZO-1), PATJ, and MUPP1 (Itoh et al., 1999; Hamazaki et al., 2002; Roh et al., 2002). Many cytosolic and nuclear proteins including regulatory proteins Rab3b, Rab13, tumor progression factors like PTEN, and transcription factors like ZONAB have been shown to interact with the tight junction complex (Nakamura et al., 2000; Wu et al., 2000; Balda et al., 2003; Yamamoto et al., 2003).

CLDNs are a large integral membrane protein family, however, only CLDN3 and CLDN4 are the family members capable of mediating *Clostridium perfringens* binding and cytolysis (Katahira et al., 1997). As a result, CLDN3 and CLDN4 are proposed to be potential targets for *Clostridium perfringens* toxin-mediated therapy for specific types of cancers. Since CCA exhibits aberrant expression of CLDN4, it is therefore possible to use CLDN4 as the target for *Clostridium perfringens* toxin-mediated therapy of CCA.

In conclusion, we demonstrated that CLDN4 is highly expressed in precancerous and CCA tissues compared to the normal bile duct epithelia and hepatocytes. Suppression of CLDN4 did not alter cell proliferation but significantly reduced cell migration and invasion of CCA cells. The molecular mechanisms to answer how CLDN4 contributes to tumor progression in CCA should be an interesting subject for future studies.

Acknowledgements

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