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CDH3/P-Cadherin regulates migration of HuCCT1 cholangiocarcinoma cells

Sungmin Baek[1,2], Yong-Whan Lee[1], Sik Yoon[1], Sun-Yong Baek[1], Bong-Seon Kim[1], Sae-Ock Oh[1,2]
[1]Department of Anatomy, School of Medicine, Pusan National University, [2]Medical Research Center for Ischemic Tissue Regeneration, Pusan National University, Yangsan, Korea

Abstract: Intrahepatic cholangiocarcinoma is the second most common subtype of primary hepatobilliary cancer. Despite advances in surgical and medical therapy, its survival rate remains poor. Compared to hepatocellular carcinoma (HCC), the most common liver malignancy, the underlying mechanisms of cholangiocarcinoma carcinogenesis are poorly characterized. P-cadherin (CDH3) is a cadherin super family member. Although CDH3 is frequently over-expressed in cholangiocarcinoma tissues, its roles have never been characterized. To determine the roles of CDH3 in cholangiocarcinoma, we investigated CDH3 function in HuCCT1 cells using specific siRNA. Transfection with *CDH3* siRNA did not affect proliferation of HuCCT1 cells. However, cell migration and invasion were significantly reduced when CDH3 was down-regulated. In addition, expressions of several biomarkers for epithelial-mesenchymal transition (EMT) were not changed by CDH3 down-regulation. These results suggest that CDH3 regulates cell migration independent of EMT in cholangiocarcinoma cells.

Key words: Cholangiocarcinoma, CDH3, migration

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Introduction

Cholangiocarcinoma is a malignant tumor originated from bile duct epithelial cells (Olnes & Erlich, 2004). Intrahepatic cholangiocarcinoma is the second most common subtype of primary hepatobilliary cancer (Kato *et al.,* 1990; Taylor-Robinson *et al.,* 1997; Olnes & Erlich, 2004; Shaib *et al.,* 2004). Between 1973 and 1997, the incidence and mortality rates of intraheptic cholangiocarcinoma in the United States was increased by approximately 9%, and is the most common primary liver cancer-related cause of death in the United Kingdom. The mortality rate of intrahepatic cholangiocarcinoma has increased in Japan, Western Europe, and Australia between 1979 and 1998. There is significant geographic variation in the incidence of cholangiocarcinoma, with the highest incidence in East Asia. This high incidence is likely due to regional risk factors, such as heptolithiasis and liver fluke infection. Despite advances in surgical and medical therapy, its survival rate is still poor. The main reasons of poor prognosis are diagnostic difficulty, extensive local tumor invasion at diagnosis and multi-drug resistance.

An important prognostic factor for cholangiocarcinoma is metastasis, which precludes curative surgical resection. Prognosis is dependent on the presence of free margins in resected tissues and the absence of lymph nodes metastasis (Olnes & Erlich, 2004). Increased cell invasion and migration is a key phenotypic advantage of malignant cells favoring metastasis. Several distinct steps have been described in the process of metastasis: detachment of tumor cells from the primary tumor, invasion into surrounding tissue, intravasation into blood or lymphatic vessels, dissemination in the blood stream or the lymphatic system, extravasation and outgrowth at a secondary site (Yilmaz & Christofori, 2009). Each of these steps requires a distinct molecular

Corresponding author:
Sae-Ock Oh
Address: Beomeo-Ri, Mulgeum-eup, Yangsan, Korea [626-870]
Tel: +82-51-510-8045, Fax: +82-51-510-8049, E-mail: hedgehog@pusan.ac.kr

program. To detach from the primary tumor and invade into the surrounding tissue, tumor cells have to break down cell-cell contacts and the remodel cell-matrix adhesion sites. These processes are known as epithelial-mesenchymal transition (EMT). During EMT, non-motile and polarized epithelial cells that are usually embedded via cell-cell junctions in a cell collective, dissolve their cell-cell junctions and convert into individual, non-polarized, motile and invasive mesenchymal cells (Yilmaz & Christofori, 2009).

Many EMT biomarkers have been reported (Zeisberg & Neilson, 2009). One of most important biomarkers is down-regulation of E-cadherin. E-cadherin is a cell-cell adhesion molecule expressed on the membrane of epithelial cells. Moreover, cancer cells undergoing EMT gain mesenchymal markers, including N-cadherin, P-cadherin, vimentin, collagen 1 and 2. EMT can be prompted by various intrinsic signals (e.g. gene mutations) as well as extrinsic signals (e.g. growth factor signaling) (Yilmaz & Christofori, 2009). Among the growth factors known to induce EMT are transforming growth factor β (TGFβ), hepatocyte growth factor (HGF), members of the epidermal growth factor (EGF) family, insulin-like growth factor (IGF) and fibroblast growth factor (FGF).

Cadherins (Calcium dependent adhesion molecules) are transmembrane proteins which play a role in cell to cell adhesion and junction (Takeichi 1995). The majority of studies on cadherins have focused on E-cadherin (CDH1) and N-cadherin (CDH2). E-cadherin is the prototype family member of classical cadherins, single-span transmembrane glycoproteins that interact in a calcium-dependent, hemophilic manner with E-cadherins on neighboring cells. E-cadherin-mediated cell-cell adhesion complexes are anchored to the actin cytoskeleton via its cytoplasmic domain, β-catenin and α-catenin. N-cadherin also forms hemophilic cell-cell adhesion junctions. It is normally expressed in nervous tissue, vascular endothelial cells and in skeletal and cardiac muscle cells. It is found to be localized in the lamellipodia and fillopodia. P-cadherin (CDH3) is one of the cadherin super family members and first identified in mouse placenta (Nose & Takeichi, 1986). In human its expression is not detectable in the placenta but is present in a few organs, such as mammary gland and prostate (Taniuchi *et al.,* 2005). Unlike the E- and N-cadherins, CDH3 has not fully been investigated and its roles remain unclear. Although previous studies have shown that CDH3 was related to various cancers, such as breast cancer (Paredes *et al.,* 2005; Paredes *et al.,* 2008; Gorski *et al.,* 2009), colorectal cancer (Milicic *et al.,* 2008; Hibi *et al.,* 2009a), gastric cancer (Hibi *et al.,* 2009b), head and neck cancer (Dasgupta *et al.,* 2006), ovarian cancer (Cheung *et al.,* 2010), and pancreatic cancer (Taniuchi *et al.,* 2005; Imai *et al.,* 2008), the pathological roles played by CDH3 remain poorly investigated.

Compared to HCC, the underlying mechanisms of cholangiocarcinoma carcinogenesis are poorly characterized. A previous study showed that CDH3 is frequently over-expressed in cholangiocarcinoma cells (Obama *et al.,* 2005). However its roles in cholangiocarcinoma cells have not yet been characterized. The aim of this study was to demonstrate the roles CDH3 play in cholangiocarcinoma cells.

Materials and Methods

Cell culture and transfection

HuCCT1 cell line was purchased from the Health Science Research Resources Bank (Osaka, Japan). HuCCT1 cells were cultured with RPMI1640, 10% FBS and 1x penicillin/streptomycin at 37°C and 5% CO_2 incubator. *CDH3* siRNA (Bioneer, Daejeon, Korea) and scrambled (SCR) siRNA (Dhamacon, Lafayette, CO, USA) were purchased. Cells were transfected with 100 nM of *CDH3* siRNA, or SCR siRNA with Dhamafect reagent (Dhamacon, Lafayette, CO, USA) in accordance with the manufacturer's protocol. SCR siRNA was used as negative control. The sequences of *CDH3* siRNA duplex were as follows: 5'-CUC UCU GGA AUG GAA CCU U-3', 5'-GAC UGA CCU ACA GUG GAC U-3', and 5'-GUG ACA ACG UCU UCU ACU A-3'

Proliferation assay

Four days after transfection of *CDH3* siRNA into HuCCT1 cells in a 96-well plate, 10 ul of Ez-Cytox (ITSBIO, Seoul, Korea) were added and incubated for 2 h under normal cell culture conditions. Cell viability was measured by absorbance at 450 nm using an ELISA reader (TECAN, Mannedorf, Switzerland).

Wound-healing assay

A day after transfection of *CDH3* siRNA into HuCCT1 cells in a 6-well plate, cells were transferred and cultured to a 48-well plate until confluent. Three hours following treatment with mitomycin C at 5 μg/ml (Sigma-Aldrich, St. Louis, MO, USA), cells were scratched using 200 ul yellow tips and

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changed fresh media to incubate for 22 h.

Matrigel invasion assay

As described by Jeon *et al.* (Jeon *et al.,* 2010), following a day of transfection with SCR or *CDH3* siRNA into HuCCT1 cells, transfected cells were seeded to a 24-well BioCoat™ Matrigel™ chamber inserts (BD Biosciences, San Jose, CA, USA). After 36 h, cells on the inside of the inserts were removed with cotton tips, and the invaded cells on the outside of the inserts were visualized using hematoxylin/eosin staining.

Real-time RT-PCR

Total RNA was extracted using RNeasy Mini kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's protocol. cDNA was synthesized with MMLV reverse transcriptase (Promega, Madison, WI, USA), dNTP and oligo-dT primers. Real-time RT-PCR was carried out using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster city, CA, USA) in the ABI Prism 7500 sequence detector (Applied Biosystems, Foster city, CA, USA) in accordance with the manufacturer's protocol. The primer sequences were as follows: CDH1 (F: 5'-TGG GCC AGG AAA TCA CAT CC-3', R: 5'-CTC AGC CCG AGT GGA AAT GG-3'), CDH3 (F: 5'-CCC CCA GAA GTA CGA GGC CCA-3', R: 5'-ACG CCA CGC TGG TGA GTT GG-3'), Fibronectin (F: 5'-GAG CTG CAC ATG TCT TGG GAA C-3', R: 5'-GGA GCA AAT GGC ACC GAG ATA-3'), SNAI1 (F: 5'-GGA CCC ACA CTG GCG AGA AG-3', R: 5'-ATT CGG GAG AAG GTC CGA GC-3'), SNAI2 (F: 5'-TTG CAA GAT CTG CGG CAA GG-3', R: 5'-AAT GCT CTG TTG CAG TGA GGG C-3'), Vimentin (F: 5'-TGA GTA CCG GAG ACA GGT GCA G-3', R: 5'-TAG CAG CTT CAA CGG CAA AGT TC-3') and β-actin (F: 5'-CAA GAG ATG GCC ACG GCT GC-3', R: 5'-TCC TTC TGC ATC CTG TCG GC-3'). β-actin was used as a loading control and all signals were normalized to β-actin.

Western blotting

Transfected cell lysates were run onto 10% SDS-PAGE gel and transferred to a PVDF membrane. Blocking was carried out with 3% BSA in PBS for 1 h at room temperature. CDH3 antibody (BD Biosciences, CA, USA) was diluted to 1 : 500 in 3% BSA in PBS, and incubated overnight at 4°C. β-actin antibody (Abcam, Cambridge, MA, USA) was diluted to 1 : 2000 in 3% BSA in PBS. HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was diluted to 1 : 2,000 in PBST and incubated for 2 h at room temperature. Blots were visualized by enhanced chemiluminescence (Amersham Bioscience, Freiburg, Germany).

Data analysis

All data are presented as means±SEM. All experiments were repeated at least 4 times. The difference between the mean values of two groups was evaluated using the Student's t-test (unpaired comparison). For comparison of more than three groups, we used one-way analysis of variance (ANOVA) test followed by Tukey's multiple comparison. A P value of <0.05 was considered statistically significant.

Results

CDH3 is knockdowned through the siRNA

Prior to investigating CDH3 functions in cholangiocarcinoma cells using siRNA, we checked whether *CDH3* siRNA specifically down-regulate its expression level. Cells

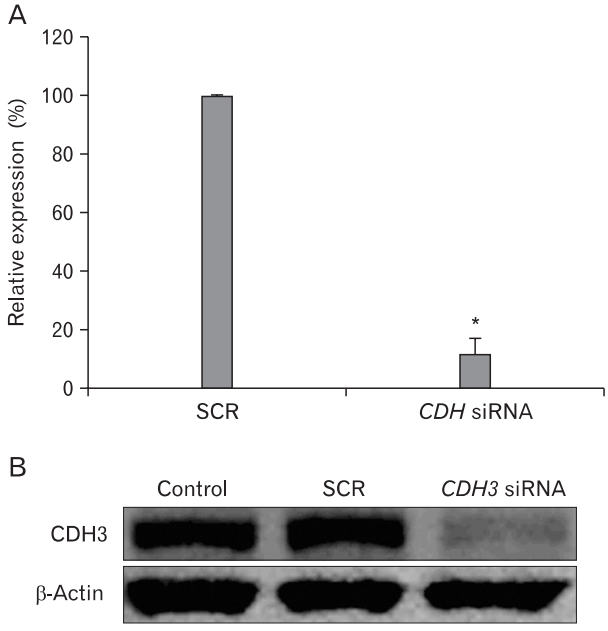

Fig. 1. *CDH3* siRNA specifically knock-downed CDH3 expression level. 48 h following transfection, CDH3 and SCR siRNA-transfected HuCCT1 cells were collected and RNA and protein were purified. Real-time RT-PCR (A) and Western blotting (B) were performed with CDH3 specific primers and antibody. β-actin was used for loading control. Data are expressed as percent change (means±SEM) compared to control. *$P<0.01$ (Student's t-test).

were harvested two days after transfection with CDH3 or scrambled siRNA. Real-time RT-PCR showed transfection of *CDH3* siRNA into HuCCT1 cells dramatically decreased its mRNA expression level while scrambled control siRNA made no impact (Fig. 1A). CDH3 protein level was measured by Western blotting using CDH3 antibody. *CDH3* siRNA significantly reduced its protein level, whereas scrambled siRNA did not (Fig. 1B). These results indicate that *CDH3* siRNA could specifically down-regulate CDH3 expression level in HuCCT1 cells.

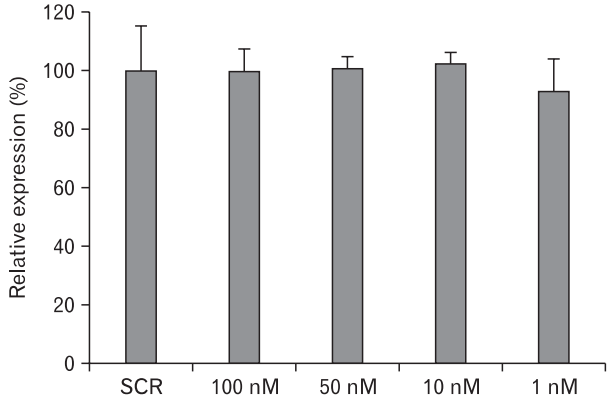

Fig. 2. Cell proliferation is not affected by down-regulation of CDH3 in HuCCT1 cells. SCR siRNA and different concentration series (100 nM, 50 nM, 10 nM, and 1 nM) of *CDH3* siRNA-transfected HuCCT1 cells in a 96-well plate were incubated for 4 d, and MTT assay was performed. Data are expressed as percent change (means±SEM) compared to control.

CDH3 has no effect on cell proliferation

Previous reports showed that CDH3 promoter is hypomethylated and highly expressed in various cancer cells and tissues (Milicic *et al.,* 2008; Hibi *et al.,* 2009a; Hibi *et al.,* 2009b). However, the roles of CDH3 in cell proliferation are poorly characterized, especially in cholangiocarcinoma cells. We therefore carried out cell proliferation assay to determine whether CDH3 affects cell proliferation in cholangiocarcinoma cells. Different concentration series of *CDH3* siRNA were transfected into HuCCT1 cells 1 d after cell seeding. Following 4 d of siRNA transfection, cell proliferation assay was performed as described in the Materials and Methods section. As shown in Fig. 2, down-regulation of CDH3 did not affect cell proliferation, consistent with a previous report (Cheung *et al.,* 2010). This data suggest that CDH3 plays in other cellular events rather than cell proliferation in cholangiocarcinoma cells.

Cell migration is decreased by CDH3 knockdown

Previous reports showed that CDH3 affects cell migration in pancreatic cancer cells (Taniuchi *et al.,* 2005) and ovarian cancer cells (Cheung *et al.,* 2010). We hypothesize that CDH3 regulates cell migration in cholangiocarcinoma cells. In order to verify this hypothesis, *CDH3* siRNA or scrambled siRNA

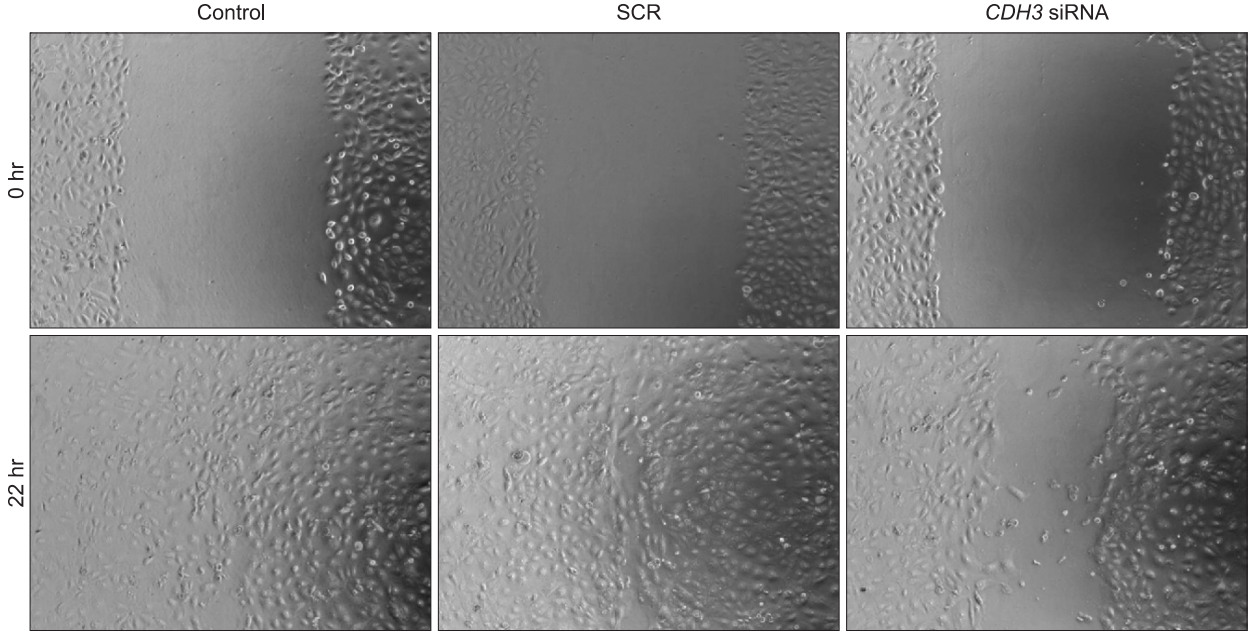

Fig. 3. Down-regulation of CDH3 in HuCCT1 cells reduced migration in wound healing assay. After transfection as described in Materials and Methods, HuCCT1 cells were scratched and allowed to migrate up to 22 h. Control = no transfected, SCR = Scrambled siRNA transfected.

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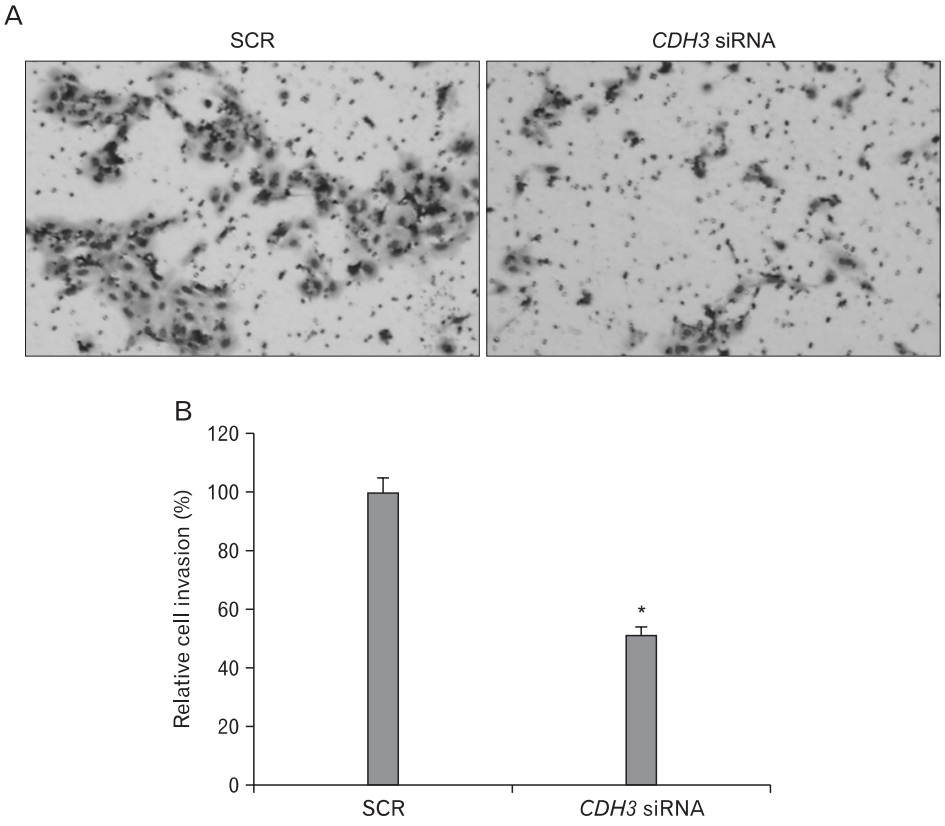

Fig. 4. Down-regulation of CDH3 in HuCCT1 cells reduced invasion in Matrigel invasion assay. After transfection as described in Materials and Methods, HuCCT1 cells were allowed to invade through the matrigel up to 36 h. Invaded cells were stained with hematoxylin/eosin (A) and counted to quantify (B). Data are expressed as percent change (means±SEM) compared to control. *$P<0.01$ (Student's t-test).

was transfected into HuCCT1 cells, and cell migration was examined using wound-healing assay. Fig. 3 shows that down-regulation of CDH3 decreased cell migration rate in HuCCT1, compared to control and scrambled siRNA. To confirm this data, we conducted matrigel invasion assay, which is a gold standard cancer cell migration and invasion assay. As shown in Fig. 4, invasion of HuCCT1 cells through the matrigel was significantly reduced when CDH3 was knock-downed. These results are consistent with previous reports (Taniuchi *et al.,* 2005; Cheung *et al.,* 2010), and suggest that CDH3 positively affect cell migration in HuCCT1 cells.

CDH3 is not related with EMT

To reveal how CDH3 regulates cell migration in HuCCT1 cells, we decided to study EMT because it can affect cancer cell migration and invasion (Yilmaz & Christofori, 2009). To determine whether EMT is involved in the effects of CDH3 on cell migration, change in expression level of EMT-related genes was measured, using real time RT-PCR after CDH3 knock-down. Fig. 5 shows that the mRNA expression levels of mesenchymal markers (snail1, snail2, and vimentin) were not significantly changed. Although the expression level of CDH1 was increased, there was no change in CDH2 expression level during CDH3 knockdown (data not shown). This result implies that CDH3-dependent cell migration may not involve EMT.

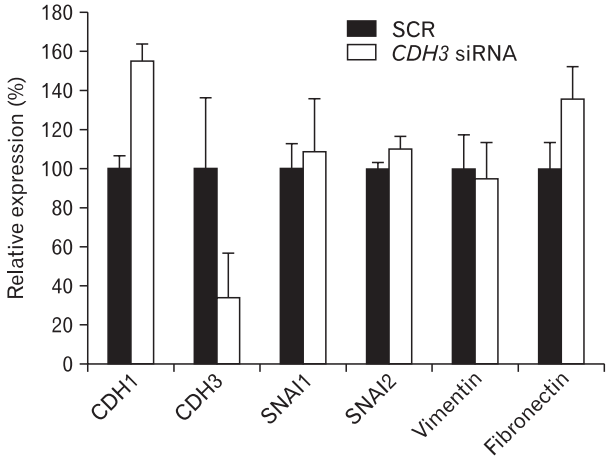

Fig. 5. Expression of EMT-related genes in CDH3-knockdowned HuCCT1 cells. RNA was purified from SCR or *CDH3* siRNA-transfected cells and real-time RT-PCR was carried out. After normalization to β-actin, data are expressed as percent change (means±SEM) compared to control.

Discussion

The carcinogenesis of cholangiocarcinoma has been poorly characterized compared to HCC. Over-expression of CDH3 in patients with cholangiocarcinoma has been shown in a previous report (Obama *et al.,* 2005). However, its roles in carcinogenesis of cholangiocarcinoma have never been examined. In the present study, we show that CDH3 regulates migration rather than proliferation in cholangiocarcinoma.

Metastatic cancers can preclude curative surgical resection and results in poor prognosis. Over-expression of CDH3 was reported to be associated with aggressive character and poor prognosis in breast and endometrial cancers (Peralta Soler *et al.,* 1999; Gamallo *et al.,* 2001; Stefansson *et al.,* 2004). Although over-expression of CDH3 in cholangiocarcinoma tissues was reported in an immunohistochemistry study (Obama *et al.,* 2005), associations between its expression and lymph node metastasis, distant metastasis or gross appearance were not observed. It must be emphasized that only 23 tumors specimens from patients were analyzed. Further studies including more tissues specimens should be conducted.

There are controversies surrounding the roles of CDH3 in cancer cells migration. CDH3 is known to promote migration in pancreatic and ovarian cancer (Taniuchi *et al.,* 2005; Cheung *et al.,* 2010). In contrast, in mammary epithelial cells, CDH3 inhibited migration (Simpson *et al.,* 2008). When Panc-1 cells were stably transfected with full-length CDH3 cDNA, cell migration was significantly enhanced although N-cadherin expression was significantly reduced (Taniuchi *et al.,* 2005). In CDH3-overexpressing Panc-1 cells, the activities of Rac1 and Cdc42 were significantly increased (Taniuchi *et al.,* 2005). Moreover, when OVCAR-3 cells and Caov-3 cells were stably transfected with CDH3 cDNA, cell migration and invasion were significantly enhanced (Cheung *et al.,* 2010). When CDH3 expression was down-regulated using *CDH3* siRNA in these cells, cell migration and invasion was significantly reduced. In contrast to these results, when CDH3 expression was down-regulated using *CDH3* siRNA in MCF-10A cells (mammary epithelial cells), cell migration was significantly enhanced (Simpson *et al.,* 2008). This discrepancy may be due to different roles CDH3 play, depending on cell type. Another explanation may be that MCF-10A cells are non-cancerous cells, whereas Panc-1, Caov-3, and OVCAR-3 cells are cancerous cells. As such, the roles of CDH3 in cancerous cells and non-cancerous cells may be different. In the present study, *CDH3* siRNA inhibited migration of cholangiocarcinoma cells in two experiments: wound healing assay, and matrigel invasion assay. The results suggest that CDH3 in cancerous cells supports cell migration.

Previous reports suggested possible mechanisms on how CDH3 regulates migration of cancer cells. Taniuchi *et al.* showed that p120-catenin was involved in the regulation of motility by CDH3 (Taniuchi *et al.,* 2005). P120-catenin is found in two forms, one bound to cadherins under the plasma membrane and the other in the cytoplasm (Kinch *et al.,* 1995; Staddon *et al.,* 1995; Anastasiadis & Reynolds, 2001). Different types of cadherins regulate cell movement by controlling the levels of p120-catenin present in the cytoplasmic pool. Taniuchi *et al.* showed that cytoplasmic accumulation of p120-catenin significantly correlated with CDH3 levels but not with the levels of E-cadherin or N-cadherin (Taniuchi *et al.,* 2005). Different distribution may be due to different affinity of p120 for each classic cadherin. Because of the low affinity for CDH3, more p120-catenin is distributed in the cytoplasm when CDH1 is replaced with CDH3 by EMT. In addition, Taniuchi *et al.* showed that activation of Rac1 and Cdc42 by CDH3 was inhibited by p120-catenin siRNA, which suggested that p120-catenin activated Rac1 and Cdc42 GTPases, which are known modulators of actin dynamics essential for cell migration and invasion (Yilmaz & Christofori, 2009).

Another mechanism on how p120-catenin regulates cell migration and invasion may involve transcriptional repressor Kaiso (van Roy & McCrea, 2005). P120-catenin can transfer to the nucleus where it binds to Kaiso. In contrast to β-catenin/Tcf-mediated transcriptions, where β-catenin acts as transactivator, p120-catenin has no transactivation domain, but rather release Kasio from its promoter binding sites, thereby activates gene expression by de-repression. However, the nature of p120/Kaiso target genes is still poorly defined (Yilmaz & Christofori, 2009).

Cadherins have been shown to play roles in signal transduction in addition to their structural roles in adhesion and migration (Pece & Gutkind, 2000; Suyama *et al.,* 2002; Qian *et al.,* 2004). For example, E-cadherin is associated with epidermal growth factor receptor (EGFR), thus activating the mitogen-activated protein kinase pathway. N-cadherin has also been found to interact with fibroblast growth factor receptor (FGFr). Moreover, interaction between CDH3 and insulin-like growth factor receptor (IGFRr) has been suggested. Furthermore, EMT is regulated by various growth factors, including EGF, FGF, HGF and IGF, whose

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signaling is finally transmitted into the nucleus where the key transcription factors, such as SNAI1, SNAI2, ZEB1 and ZEB2 are induced (Thiery *et al.,* 2009; Yilmaz & Christofori, 2009). These results suggest that a change in CDH3 expression level may modulate EMT induced by a certain growth factor. To test this hypothesis, we examined change in the expression of EMT marker genes following transfection with *CDH3* siRNA. However, we did not observe consistent changes in EMT marker genes expression (Fig. 5).

Although other EMT marker genes including SNAI1, SNAI2, and vimentin were not significantly changed after transfection with *CDH3* siRNA, the expression of CDH1 was increased, which may suggest redundancy in the level of cadherins (Fig. 5). During EMT in carcinogenesis, cadherin switch has been well documented (Zeisberg & Neilson, 2009). However, effects of change in the expression of CDH3 on other cadherins expression were different in different cells. The expression level of CDH1 or CDH2 was not changed by over-expression or knock-down of CDH3 in ovarian cancer cells (Cheung *et al.,* 2010). The expression level of CDH2 was reduced however that of CDH1 was not changed by over-expression of CDH3 in pancreatic cancer cells (Taniuchi *et al.,* 2005).

The roles of cadherins in signal transduction pathway indicate that they can regulate proliferation. N-cadherin negatively controls osteoblast proliferation and survival via inhibition of autocrine/paracrine Wnt3a ligand expression and attenuation of Wnt, ERK and PI3K/Akt signaling (Haÿ *et al.,* 2009). E-cadherin can negatively regulate, in an adhesion-dependent manner, ligand-dependent activation of divergent classes of receptor tyrosine kinases (RTKs), by inhibiting their ligand-dependent activation in association with decreases in receptor mobility and in ligand-binding affinity (Qian *et al.,* 2004). As such, E-cadherin neutralizing antibody inhibited proliferation of MDCK cells. However, according to published reports, CDH3 does not regulate cell proliferation, although CDH3 is highly expressed in numerous cancer tissues (Taniuchi *et al.,* 2005; Milicic *et al.,* 2008; Cheung *et al.,* 2010). In Panc-1 cells, neither over-expression of CDH3 nor functional-blocking of CDH3 regulated cell proliferation (Taniuchi *et al.,* 2005). Moreover, when OVCAR-3 cells and Caov-3 cells were stably transfected with CDH3 cDNA, cell proliferation was not affected (Cheung *et al.,* 2010). Furthermore, the crypt fission rate was not significantly increased in CDH3 transgenic mouse that harbored CDH3 over-expression in the intestinal and colonic epithelium (Milicic *et al.,* 2008). Consistent with these results, the present study showed that down-regulation of CDH3 did not affect proliferation in cholangiocarcinoma cells.

In summary, CDH3 regulates cell migration through EMT-independent signaling in cholangiocarcinoma cells. Our data will contribute to the development of diagnostic molecular markers and therapies against cholangiocarcinoma.

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