The Role of CD44v6 Isoform in Cholangiocarcinoma Invasiveness

ศึกษาหน้าที่ ของCD44v6ในมะเร็งท่อน้ำดี

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ABSTRACT

CD44v6, a splice variant of the CD44 cell surface protein family, has been implicated in adhesion, chemoresistance, invasion and metastasis of many cancer types. Besides, CD44v6 can serve as a coreceptor for the c-Met and VEGFR-2 receptors whose signaling requires coupling of CD44v6 to the cytoskeleton via ezrin. Cholangiocarcinoma (CCA) is a lethal disease originated from the bile duct epithelium. It is one of the most common causes of cancer-associated death in Thailand due to high rate of growth and metastasis. Here we investigated the role of CD44v6 in CCA using HuCCA-1 cell line as a model. HuCCA-1 expressed moderate level of CD44v6 protein as determined by western blot. Silencing of CD44v6 reduced the protein levels by 50%, compared to cells transfected with negative control siRNA. *In vitro* invasion, but not migration, as determined by Transwell assay, showed a 25% suppression. Moreover, the ability to close the wound in the wound-healing assay was significantly compromised. Our findings suggest that CD44v6 plays an important role during metastasis of CCA and may serve as a potential target for CCA treatment.

บทคัดย่อ

CD44v6 เป็นโครงสร้างที่ผันแปรของ CD44 ซึ่งเป็น receptor โมเลกุลที่อยู่บนผิวเซลล์ CD44v6 เป็นโมเลกุลที่ทำหน้าที่ช่วยเหลือกระบวนการต่างๆของมะเร็งชนิดต่างไม่ว่าจะเป็น การยึดเกาะต่อ Matrigel, การดื้อยา และ การบุกรุก จากงานวิจัยที่ได้ทำการศึกษาบทบาทของ CD44v6 ในมะเร็งท่อน้ำดีพบว่า CD44v6 ยังทำหน้าที่เป็น coreceptor ร่วมกับ c-Met และ VEGFR-2 receptors ซึ่งจะทำการส่งสัญญาณผ่านการทำงานร่วมกับ CD44v6 ผ่าน ezrin ไปให้ cytoskeleton ในงานวิจัยนี้ได้ทำการศึกษาบทบาทของ CD44v6 ในมะเร็งท่อน้ำดี คือ HuCCA-1 โดยการ knock down ยีน CD44v6 ด้วย siRNA พบว่า CD44v6 protein มีการแสดงออกหลัง knock down ลดลงถึง 50% เมื่อเทียบกับ negative siRNA การยับยั้งการแสดงออกนี้สามารถที่จะลด ความสามารถในการบุกรุก ได้อย่างมีนัยสำคัญ แต่ไม่มีผลต่อการเคลื่อนที่ของเซลล์ และการยับยั้งการแสดงออกนี้ยังสามารถจะลด ความสามารถในเคลื่อนที่แบบ non-chemotactic ได้อย่างมีนัยสำคัญ การศึกษาทั้งหมดนี้แสดงให้เห็นว่า CD44v6 มีความสำคัญต่อกระบวนการแพร่กระจายของเซลล์มะเร็งท่อน้ำดี และสามารถนำไปพัฒนาให้เป็น potential target ในการรักษามะเร็งท่อน้ำดี

Key Words: CD44v6, Cholangiocarcinoma, Invasiveness

คำสำคัญ: ซีดี44วี6 มะเร็งท่อน้ำดี กระบวนการบุกรุก

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Introduction

Cholangiocarcinoma is the malignant tumor of bile duct arising from epithelial cells lining along the biliary tract from small peripheral intrahepatic to extrahepatic bile duct. Although cholangiocarcinoma is a rare disease worldwide, the incidence rate of CCA in Thailand is the highest in the world and is correlated with Opisthorchis viverrini infection. CCA is a lethal disease with 5-year survival rate of less than 5% and 75% of individuals afflicted with this disease died within 1 year of diagnosis.

The CD44

The families of proteins known collectively as cell adhesion molecules are cell surface glycoproteins. These molecules are termed adhesion molecules because of their relatively strong bonding to specific ligands. However, the interaction is more complex than cell attachment, and may be more involved with the cell "sensing" the extracellular environment and sending out information to adjacent cells. Cell adhesion proteins are involved in many functions such as cell-cell interaction, cell-matrix interactions, cell migration, cell differentiation, cell signaling and gene transcription. Moreover, the expression patterns of cell adhesion molecules are being increasingly implicated in disease processes and they are candidates for use in diagnostic pathology(1). The proximal extracellular domain of CD44 is the site of alternative splicing for CD44 mRNA that produces different variant isoforms of CD44. While the standard isoform of CD44 (CD44s) is expressed predominantly in normal epithelial cell subsets, CD44 variant isoforms (CD44v), which contain additional insertions, are highly expressed in many epithelial-type carcinomas. Expression of CD44v has been closely linked to tumor progression, metastasis, and treatment resistance processes in various cancers. In articular, the variant CD44 molecule containing exon 10, also known as CD44 variant 6 (CD44v6)

CD44v6, a splice variant of the CD44 cell surface protein family, serves as a co-receptor of c-Met and VEGFR-2 receptors whose signaling require coupling of CD44v6 to the cytoskeleton via ezrin(2). CD44v6 has been implicated in adhesion, chemoresistance, invasion and metastasis of many cancer types, such as breast cancer, prostate cancer and gastric cancer. Silencing of CD44v6 has been shown to decrease adhesion and migration of ovarian cancer, breast cancer and hepatocellular carcinoma(3-7). Study in cholangiocarcinoma using immunohistochemicalanalysis indicated that CD44v6 expression was correlated with short survival of CCA patients and was implicated in early carcinogenesis as well as cancer progression(8).

Objectives

1 . To determine the expression level of CD44v6 in cholangiocarcinoma cell lines.

2 . To determine the biological roles of CD44v6 in cholangiocarcinoma cells, using siRNA-mediated silencing.

3 . To determine the effects of CD44v6-mediated silencing on the downstream signaling pathways.

Methodology

Cell culture

Ham's F-12 medium and fetal bovine serum (FBS) were purchased from Gibco (Gibco, Grand Island, NY, USA). The human CCA cell lines, HuCCA-1 and KKU100, were grown in HAM's F-12

medium upplemented with 10% FBS at 37^{O}C in a 5% CO_2 humidified atmosphere.

Small interfering RNA (siRNA) for inhibition of CD44v6 expression

The siRNA sequences used for silencing of CD44v6 gene was purchased from Invitrogen. The sequences of sense and antisense of CD44v6 siRNA were

Sense: 5'GCAACUCCUAGUAGUACAAtt 3'

Antisense:

5'UUGUACUACUAGGAGUUGCtt 3'

For a negative control, Silencer® Negative control (Ambion) was used which has no homology to any mammalian gene sequence and comprise of a 19 bp scrambled sequence with 3' dT overhangs.

Western Blot analysis

For western blot analysis, 3.5x10^5 HuCCA-1 cells

were seeded in a 60 mm culture dish for 24 h before transfection with siRNA. For KKU-100, 3x10^5 cells were mixed with the siRNA complexes immediately before seeding into the culture dish. At 48 and 72 h post-transfection, respectively, HuCCA-1 and KKU-100 cell lysates were suspended in lysis buffer, composed of 50 mM Tris-Cl pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 2 mM Na_3VO, 50 mM NaF, 50 mM β-glycerophosphate, protease inhibitor cocktail and DTT. Protein samples were mixed with SDS sample buffer and β-mercaptoethanol, boiled and 30 µg of protein were separated using 10% SDS-PAGE. Gel were run for 2 h at 120 V before the proteins were transferred onto a nitrocellulose membrane by electroblotting for 2.5 h at 120 V, 4^{O}C. After that the membrane was incubated with 5% non-fat dry milk in TBST (10mM Tris-Cl pH 8.0, 150 mMNaCl and 0.1% (v/v) Tween 20) for 1 hour at room temperature to block nonspecific protein binding. The primary antibody, anti-CD44v6 was diluted 1:1000 in 10% skim milk in TBST. The blot was probed overnight at 4ºC. Next day, membrane was washed 3 time (10 minutes each time) in TBST. A donkey anti-mouse IgG secondary antibody conjugated with horseradish peroxidase at 1:2000 dilution in TBST was used as secondary antibody and incubated for 1 hour at room temperature. Next, the membrane was washed 3 times with TBST. The immunoreactive bands were visualized by enhanced chemiluminascence (ECL, BIO-RAD).

Cell invasion assay

At 48 h (HuCCA-1) post-transfection cells were harvested for in vitro invasion assay, which was performed in a 24 well Transwell chamber with 8 µm pore filters (Transwell, Coster, Boston, MA). The suspension of 1x10^5 cells in Ham's F-12 without FBS was seeded in the upper chamber coated with 30 µg Matrigel (BD Biosciences, Bedford, MA, USA) per well, while 600 µl of medium with 10% FBS was added in the lower chamber as a chemoattractant. After 6 h of incubation at 37^{O}C in a 5% CO atmosphere, cells on the upper surface of the Transwell were removed using cotton swabs, while the cells that had invaded into the lower surface were fixed in methanol and stained with 0.5% crystal violet. The invaded cells of each well were counted to whole well under light microscope at 10x magnification.

Migration assay

In vitro Cell migration assay was performed in the same manner as in vitro invasion assay except that Matrigel coating was omitted.

Wound healing assay

Horizontal migration abilities of HuCCA-1 cell was measured using the *in vitro* wound healing assay. $3x10^5$ of HuCCA-1 cells were transfected and seeded onto a 6 well-plate. HuCCA-1 was cultured in HAM's F-12 medium supplemented with 10% FBS for 48 hours at 37ºC with 5% CO_2 in humidified incubator. At the end of transfection, wound were created by scraping the monolayer of cells with a sterile pipette tip (200 µl). Detached cells after scraping were removed by washing with PBS. Next Ham's F-12 medium supplemented with 10% FBS was added in the culture dish for 2.5 ml. Hereafter cells were allowed to migrate into the cell-free area, and photographs were taken at various time points on a phase-contrast microscope with moticam 1000 software application.

Results

Expression of CD44v6 proteins in CCA cell lines

In this project, we examined the protein pattern of CD44v6 in the cholangiocarcinoma cell lines, HuCCA-1, KKU-100, RMCCA and KKU-M213 cell lines. The protein patterns of CD44v6 were determined by western blot assay. The results of western blot showed that multiple protein bands were hybridized by the CD44v6 antibody. This include 195, 128, 108, 93 and 83 kDa, etc. bands. Although based on amino acid sequence CD44v6 is calculated to display a size of 47 kDa, high molecular weight bands are observed due to multiple post-translational modifications such as glycosylation, methylation, phosphorylation. HuCCA-1 had the highest expression of CD44v6 when compared to the other cholangiocarcinoma cell lines. (Figure1 A, B). The expression levels from the highest to the lowest are as follows: HuCCA-1>KKU-M213>RMCCA-1>KKU-100.

Silencing of CD44v6 expression by siRNA

HuCCA-1 cells was transfected with CD44v6 siRNA or negative control siRNA before the efficiency of siRNA- mediated gene silencing was determined by Western Blot analysis. CD44v6 expression in HuCCA-1 cells was efficiently suppressed by 20 nM CD44v6 siRNA at 48 hours post-transfection. The upper 80 kDa bands were significantly downregulated, showing a 75.98± 7.93% suppression at 48 hours post-transfection compared to cells transfected with negative control siRNA(Figure 2 A and B.). In addition, the double bands at 80 kDa was slightly suppressed, therefore they are likely the non-specific bands. Hereafter, we performed phenotypic assessment of the silenced HuCCA-1 cell at 48 hours post-transfection.

Effects of CD44v6 silencing on cell invasion and migration

In cholangiocarcinoma, previous report using immunohistochemical analysis indicated that CD44v6 expression was correlated with short survival of CCA patients and was implicated in early carcinogenesis as well as cancer progression, (8). However, no previous reports had described the roles of CD44v6 on the metastatic properties of cholangiocarcinoma. Hence, we designed to determine the role of CD44v6 on the metastatic properties of cholangiocarcinoma, using CD44v6

siRNA. The result showed that, CD44v6 silencing significantly reduced *in vitro* invasiveness of HuCCA-1 compared to negative control, suggesting that CD44v6 is required for the invasiveness of HuCCA-1 cell. *In vitro* invasion of HuCCA-1 was reduced by 25 ± 6% , compared to negative control. (Figure 3)

Since invasion is a multi-step process comprising of cell adhesion, migration and MMP secretion, defect in any of these processes may affect the invasiveness of the cancer cells. The above data showed that silencing of CD44v6 significantly reduced cell invasion. Hence, next determine if this reduction was due to impairment of cell migration. As shown in Figure 4, CD44v6 silencing did not significantly reduce *in vitro* migration of HuCCA-1 cell compared to negative control.

Effect of CD44v6 silencing on wound healing

Since our data showed that silencing of CD44v6 did not reduce cell migration of HuCCA-1 as determined by *in vitro* Transwell assay, we confirmed that this was the case by using the wound-healing assay. Unlike the Transwell assay which determines vertical mobility of cells in the presence of chemotactic gradient, the wound-healing assay measures horizontal mobility of cells in the absence of chemotactic gradient. In this assay, HuCCA-1 was transfected with CD44v6 siRNA or negative control siRNA for 48 hours before the wound was created. Data showed that, the migration rate of HuCCA-1 cells transfected with CD44v6 siRNA was reduced (Figure 5) by 25.4±2.94% , compared to negative control.

Since the extent of reduction of *in vitro* invasion by Transwell assay and that of the horizontal migration by wound-healing assay was similar (25±6%, versus 25.4±2.934% reduction, respectively, compared to negative control (** $P< 0.01$)), these data suggest that CD44v6 is essential for invasion and horizontal migration of cholangiocarcinoma cells.

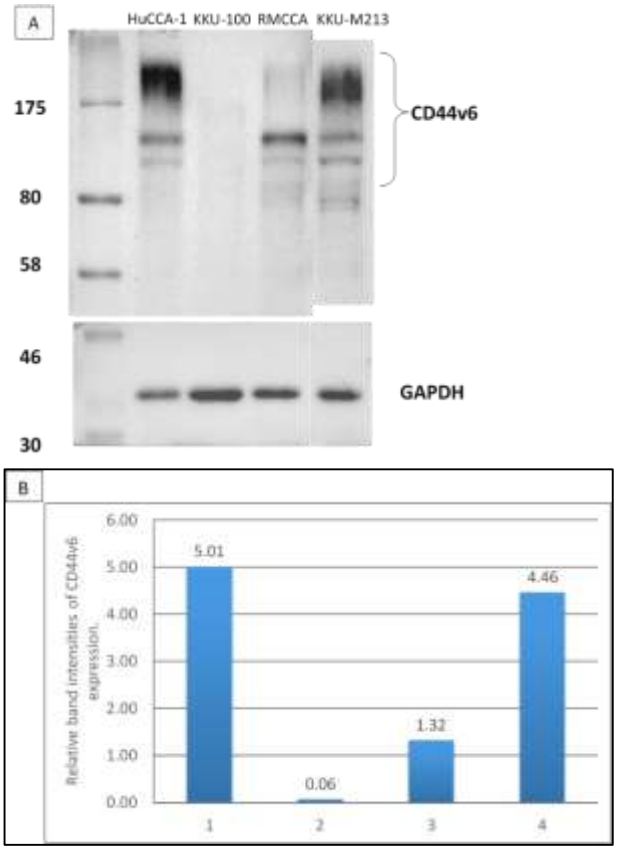

Figure 1 A. Expression of CD44v6 protein in cell lysate of cholangiocarcinoma cell lines as determined by western blot analysis. The blot was probed with monoclonal anti-CD44v6 (upper panel). The blot was probed with GAPDH (lower panel) to normalize the loading of proteins in all lanes. B. CD44v6 intensities on the western blot were quantified and presented as a relative intensities in four cholangiocarcinoma cell lines. Data are showed as Mean ± SEM of results expressed as percentage of CD44v6 band

intensities from three independent experiments.

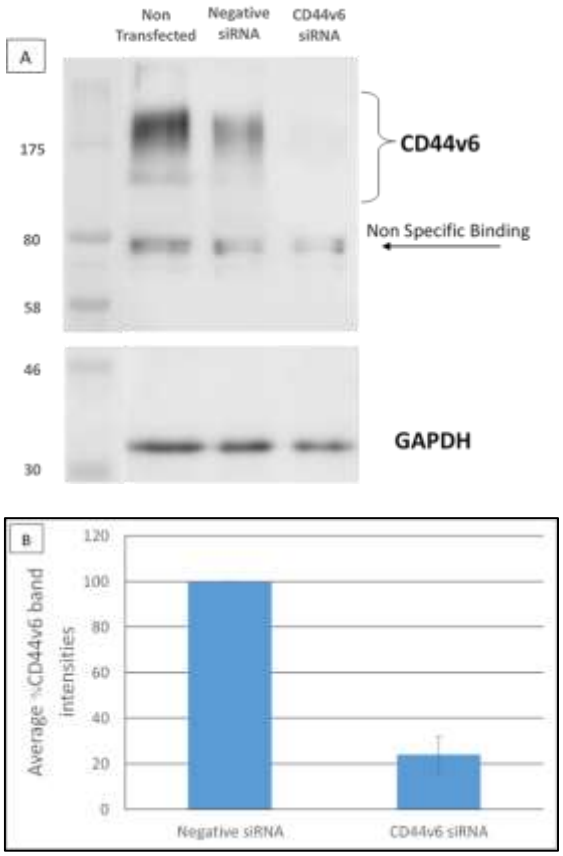

Figure 2 A. CD44v6 siRNA significantly suppressed the expression of CD44v6 protein at 48 hours post-transfection. 30µg of cell lysate from the transfected cells at 48 hours were analyzed in a 10% SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed with anti-CD44v6 antibody (upper panel) and probed with anti-GAPDH antibody (lower panel) to normalize the loading of proteins in all lanes. B. Western blot band intensities were quantified and presented as a percentage of negative control siRNA. Data are showed as Mean ± SEM of results expressed as percentage of CD44v6 band intensities from three independent experiment compared with negative control.

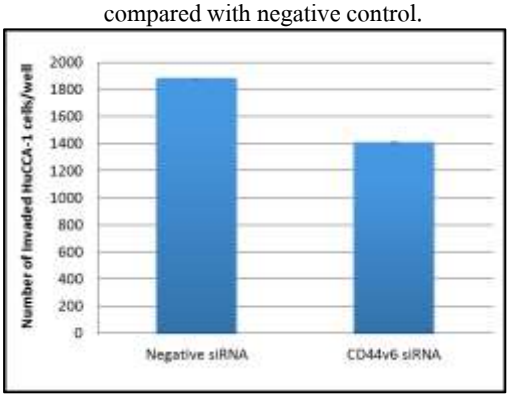

Figure 3 Transwell assay to determine the effect of CD44v6 on *in vitro* invasion of HuCCA-1 cell line. Cell suspension of HuCCA-1 cells ($1x10^5$ cells) transfected with CD44v6 siRNA or negative control siRNA were seeded onto the upper chamber of the Matrigel-coated Transwell. After 6 hours of incubation, invaded cells that adhered to the lower surface of the membrane were fixed, stained with crystal violet, and counted under a microscope. The average number of cell invasion (Y-axis) was plotted.

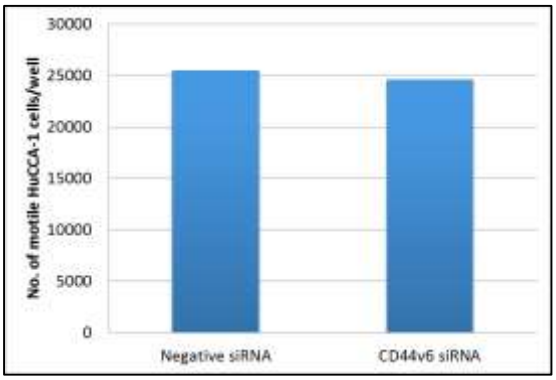

Figure 4 Transwell assay to determine the effect of CD44v6 on in vitro migration in HuCCA-1 cell line. Cell suspension of HuCCA-1 cells ($5x10^4$ cell) transfected with CD44v6 siRNA or negative control siRNA were seeded onto the upper chamber of Transwell without Matrigel coating. After 6 hours of incubation, migration cells that adhered to the lower surface of the membrane were fixed and stained with crystal violet, and counted under a light microscope. The

average number of cell migration (Y-axis) was plotted. From this result shown that migration of cells transfected with CD44v6 siRNA was not significantly different compared to that of cells transfected with negative control siRNA.

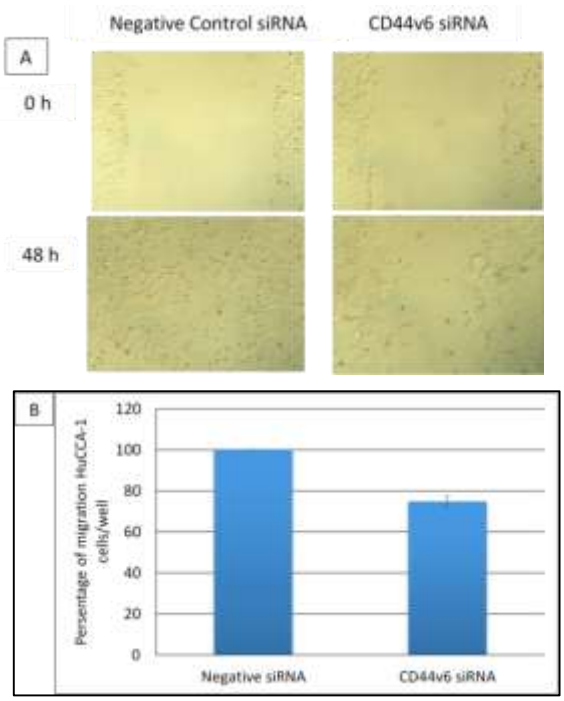

Figure 5 A. Wound-healing assay to determine the effects of CD44v6 silencing on horizontal cell migration. Cell suspension of HuCCA-1 cells ($3x10^5$ cells) transfected with CD44v6 siRNA or negative control siRNA were grown to confluency before a linear wound was created with a pipette tip. Photographs were taken at 0 and 48 h after wounding. B. Effects of CD44v6 silencing on cell migration were presented as a percentage of negative control siRNA.

Conclusion

From all of that, we concluded that HuCCA-1 was the highest expressed CD44v6 protein levels which it was down regulated with siRNA-mediated silencing. From western blot result which showed multiple bands to CD44v6 antibodies, suggesting multiple CD44v6 isoforms as a result of alternative splicing and post translational modifications including glycosylation. Moreover, CD44v6 may regulate the metastatic properties by directly linking with the cytoskeleton and/or signaling molecules. Alternatively, CD44v6 may play an indirect role by serving as a co-receptor for c-MET and VEGFR-2, both of which are well-known key players in cancer metastasis. Finally, CD44v6 plays a crucial role during metastasis of CCA, and may serve as a potential target for CCA treatment.

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