Expression and Possible Role of CD44 in Cholangiocarcinoma Cell Line.

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ABSTRACT

Cholangiocarcinoma is a cancer of the bile duct and is a major health problem in the north-eastern part of Thailand. CD44 is a family of cell surface adhesion molecule, consisting of numerous members generated by alternative splicing of a single gene. Expression of CD44 variant isoforms has been implicated in tumorigenesis as well as in the invasiveness and metastatic potentials of many tumor types. Although CD44 has been shown to bind to many ligands, the major one is hyaluronic acids (HA), a sugar which is a component of the extracellular matrix (ECM). In this project, we investigated if CD44 plays a role in regulating the metastatic property of cholangiocarcinoma, using a cell line, KKU-M213, as a model. We showed that CD44 was expressed in KKU-M213 using RT-PCR and immunofluorescence microscopy. Furthermore, we demonstrated that the invasiveness of KKU-M213 was dependent on HA concentrations, suggesting that this process was mediated by CD44.

Using RT-PCR and primers specific to standard exons 5 and 16, which are present in all CD44 transcripts, two weak bands of 569 bp and 965 bp were detected, whereas immunofluorescence staining of live cells with CD44-FITC polyclonal antibodies specific to all CD44 forms showed weak expression at the cell membrane of KKU-M213. In vitro invasion assay revealed that increasing concentrations of HA (50-200 µg/ml) significantly enhanced invasion of KKU-M213 cells through artificial extracellular matrix (Matrigel), where 50 µg/ml HA augmented the invasiveness of KKU-M213 cells by 70 percent compared to control (no HA). However, the invasiveness dropped at higher concentrations HA (100 µg/ml and 200 µg/ml, respectively). These data indicated that the KKU-M213 cells expressed CD44 which are likely to be responsible for the HA-dependent activation of the cancer cell invasiveness.

Keywords: CD44, cholangiocarcinoma, extracellular matrix, hyaluronic acid, invasion

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Introduction

Cholangiocarcinoma is a malignancy of the bile duct epithelium. The tumor arises from the ductular epithelium of the biliary tree, either within the liver (intrahepatic cholangiocarcinoma) or more commonly from the extrahepatic bile ducts (extrahepatic cholangiocarcinoma) (Lazaridis and Gores, 2005; Khan et al., 2005). In most cases of cholangiocarcinoma, the stimulating cause is unknown, but chronic inflammation and cellular injury within bile ducts, together with partial obstruction of bile flow, exhibited in high-risk condition for cholangiocarcinogenesis such as primary sclerosing cholangitis (PSC), hepatolithiasis and liver fluke infestation by Opisthorchis viverrini or Clonorchis sinensis, Caroli’s disease and congenital choledochal cysts, clearly seem to be relevant predisposing factor in pathogenesis of cholangiocarcinoma. The prevalence of cholangiocarcinoma is the highest in regions of East Asia where O. viverrini are endemic. In these areas, infestations with the liver flukes are associated with intrahepatic stones and cholangiocarcinoma (Sirica AE., 2005). Cholangiocarcinoma is particularly common in the northeastern region, and has been associated with infection by liver fluke Clonorchis sinensis and Opisthorchis viverrini. The habit of eating uncooked cyprinoid fish which are infected with the flukes underlies the high prevalence of cholangiocarcinoma in northeastern Thailand (Watanapa, P. and Watanapa, W.B., 2002; Vatanasapt et al., 2002; Sripa and Pairojkul, 2008).

Cholangiocarcinoma exhibits a high mortality rate due to the late clinical presentation and the lack of effective nonsurgical therapeutic modalities. Most patients have unresectable disease at presentation and usually die within 12 months. Overall survival rate, including resected patients, is poor, with less than 5% of patients surviving to 5 years, a rate which has not changed significantly over the past 30 years. Moreover, cholangiocarcinoma is associated with high rates of local and distant metastasis (Anderson et al., 2004). Ashida K. et al., 1998 showed that CD44, a cell surface adhesion molecule, was not expressed at the cell membrane of normal intrahepatic bile ducts. Instead, CD44 was frequently expressed at the membrane of cholangiocarcinoma cells and much less frequently in the cholangiocarcinoma cytoplasm. It was suggested that CD44 was neoexpressed during carcinogenesis of cholangiocarcinoma (Ashida et al., 1998).

CD44 is a type I transmembrane cell surface glycoprotein consisting of a single polypeptide chain. It is encoded by a single gene, with alternative splicing providing the potential for multiple isoforms. The human CD44 gene has been mapped to the chromosomal locus 11p13 and is composed of two groups of exons. One group, comprising exons 1–5 and 16–20, are spliced together to form a transcript that encodes the ubiquitously expressed standard isoform (abbreviated as CD44s). The 10 variable exons 6–15 (also known as v1–10) are alternatively spliced and included within the standard exons at an insertion site between exons 5 and 16. CD44 is expressed throughout development on many different cell types including hematopoietic cells, fibroblasts, some epithelial and endothelial cells, and cells in the central nervous system (Goodison et al., 1999). Changes in CD44 expression, including up-regulation, down-regulation and changes in the isoforms, have been associated with tumor progression, invasion, and metastasis as well as with patients survival. However, some reports did not find any relationship between CD44 expression and tumor behavior. Proposed functions of cell surface CD44 include participation in cell adhesion and migration, lymphocyte activation, assembly of extracellular matrix (ECM), and metastatic behavior of tumor cells (Marhaba and Zoller, 2004). CD44 and its principal ligand, hyaluronic acid (HA), are broadly distributed in the body. The expression of multiple CD44 isoforms and the resulting HA binding profile can influence tumor growth and development (Pure and Cuff, 2001). The role of CD44 has been directly demonstrated by silencing of CD44 expression using siRNA in breast,
prostate and colon cancer (Draffin et al., 2004; Subramaniam et al., 2007). Although the expression pattern of CD44 has been examined in cholangiocarcinoma, whether it has a role in regulating cholangiocarcinoma growth or invasiveness has not been determined.

**Objective**

1. To examine CD44 expression patterns in KKU-M213 cell line, both at the mRNA and protein levels.

2. To determine if CD44 mediates HA-dependent invasiveness of KKU-M213 cells.

**MATERIALS AND METHODS**

**Part I: Cell culture**

KKU-M213 cell line was established from a patient with adenosquamous carcinoma by Dr. Banchob Sripa, Department of Pathology, Faculty of Medicine, Khon Kaen University.

**Maintenance of cell lines and cell culture:** KKU-M213 cells were grown as a monolayer in HAM’s F-12 medium, supplemented with 10% heat-inactivated fetal bovine serum (FBS). The cell cultures were incubated under 5% atmosphere at 37°C and subcultured two to three times per week.

**Determination of cell number:** The numbers of cells were counted by using trypan blue dye exclusion methods. The basis of this technique is that, living cells are able to exclude trypan blue, while dead cells cannot. Thus live cells would appear white, while dead cells would appear blue. After staining, the medium was removed from culture and rinsed twice with Phosphate Buffer Saline (PBS) pH 7.4. Cells were harvested using 1 ml trypsin-EDTA solution (0.25% trypsin-0.02% EDTA) and incubated at 37°C for 3-5 minutes or until cells detached. Fresh medium was added and drawn up and down with pipette until cells were completely dispersed. The cell suspension was transferred to a chamber of hematocytometer. The viable cells were counted in the five corners. The number of cells (cells/ml) was calculated by: (Total number of cells in 5 corners / 5) × 10⁴ × dilution factor

**Part II: To study CD44 mRNA and protein expression in cholangiocarcinoma cell line**

**RNA preparation:** 10⁶ cells were seeded on to a culture dish and incubation for 24 hours before being washed in PBS, trypsinized and collected from culture dish. Total cellular RNA was extracted using RNAspin Mini kit (GE Healthcare), quantitated and assessed for the purity by spectrophotometry.

1 A260 unit = 40 µg/ ml (single strand RNA)

**Reverse transcriptase reaction (RT-PCR):** One microgram of total RNA was used in each reaction for all samples. All RT reactions were carried out with oligo-dT priming to target transcription of polyadenylated mRNA. The reaction was performed at 70°C for 5 minutes followed by an incubation at 4°C for 5 minutes. Then RT reaction master mix was added into the RNA target and incubated at 25°C for 5 minutes, 42°C for 60 minutes, and followed by the termination reaction at 70°C for 15 minutes. cDNA was stored at -20°C before performing PCR reaction.

**Polymerase chain reaction (PCR):**

To detect CD44 transcripts, PCR reaction was performed using 1 µl of cDNA as a template, and forward CD44 primer (5’ GAT GGA GAA AGC TCT GAG CAT C 3’) and reverse CD44 primer (5’ TTT GCT CCA CCT TCT TGA CTC C 3’) specific for exons 5 and 16, respectively. To detect β-actin transcript, PCR reaction was performed using 1 µl of cDNA, and forward β-actin primer (5’ TCT TCC AGC CCT CTT CCT C T 3’) and reversed β-actin primer (5’ AGC ACT GTG TTG GCG TAC AG 3’). The PCR reaction was set up as follows: 1 cycle of denaturing at 95°C for 5 minutes followed by 25 cycles of 94°C for 60 seconds, 56°C for 60 seconds and 72°C for 90 seconds, before a final primer sequence extention incubation at 72°C for 5 minutes. RT-PCR reactions were set up in Minicycler™ model PTC-1152. PCR amplicons were

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analysed by running on a 2% agarose gel electrophoresis

**Fluorescent staining of live cells:** $3 \times 10^5$ cells were seeded on coverslips in a 35-mm dish for 24 hours. The next day, cells on the coverslip were washed with HAM’s F-12, blocked with 4% bovine serum albumin (BSA) in HAM’s F-12 for 1 hour, and incubated with fluorescein isothiocyanate (FITC) anti-mouse/human CD44 at a dilution of 1: 100 in 1% BSA in HAM’s F-12 for 1 hour. After that, cells were washed and fixed with 4% paraformaldehyde in PBS for 15 minutes, followed by a nuclear staining with TOPROIII (1: 5000) for 1 hour. After washing, coverslips were mounted with Slow-Fade in glycerol-PBS (Molecular Probes). Immunofluorescence signals were analysed by confocal fluorescent microscopy.

**Part III:** Biological assay

**In vitro invasion assay:** Invasion assays were performed using Transwell with a 6.5 mm diameter polyvinyl pyrolidone (PVP)-free polycarbonate filter of 8 μm pore size. The filter was coated with 30 μg Matrigel in 100 μl serum free medium, mixed with 0, 50, 100, and 200 μg/ml of HA. Matrigel (with or without HA) was left to gel to form a thin layer at 37°C, 5% CO₂ in an incubator overnight. The next day, the Matrigel layer was reconstituted with 100 μl of serum free medium at room temperature for 90 minutes at 37°C. Excess medium was removed from the filters prior to use. Cancer cells were freshly harvested by trypsinization, and viable cell were counted before 1x10⁵ cells suspended in culture medium without FBS were added to the upper compartment of the Transwell. The lower chamber contained 600 μl of culture medium containing 10% FBS. After 6 hours of incubation at 37°C, 5% CO₂ atmosphere, non-invasive cells on the upper surface of the filter were wiped away using cotton swabs. The cells that had invaded into the lower surface were fixed with 25% methanol for 30 minutes, stained with 0.5% crystal violet for 1 hour, and destained with tap water 2-3 times. The invaded cells in each well were counted in five fields under a light microscope.

**RESULTS AND DISCUSSIONS**

Using primers specific to exons 5 and 16 which are common to all CD44 transcripts, at least two weak bands of 569 bp and 965 bp were detected in KKU-M213 using RT-PCR and agarose gel electrophoresis (Fig. 1). The expected size of amplicon from the CD44s transcript is 569 bp. The 965 bp was likely generated by inclusion of one or more variable exons. Our data suggested that KKU-M213 cell line expressed CD44 mRNAs, corresponding to the standard form and at least 1 variable isoform of CD44.

We also examined the expression of CD44 protein by immunofluorescence staining of live cells using a polyclonal CD44-FITC antibody specific to all forms of CD44. As shown in Fig. 2, green staining corresponding to CD44 protein was detected on the cell membrane, although the signal was quite weak. Consistent with RT-PCR data, KKU-M213 expressed low level of CD44 protein on the cell membrane.

Since CD44 protein was detected in KKU-M213 cell membrane, we tested if CD44 was involved in the invasiveness of this cell line by assessing the **in vitro** invasiveness of KKU-M213 in the presence of various concentrations of HA. Since HA is the principal ligand of CD44, we expected that increasing concentrations of HA would result in a corresponding increase of invasiveness. Our data showed that 50 μg/ml of HA resulted in a 1.7 folds increase of **in vitro** invasion of KKU-M213 cells compared to control (the condition without HA). Stimulation by higher concentrations of HA (100 and 200 μg/ml, respectively) also increased the invasiveness compared to control, although the extent of the increase was reduced compared to that stimulated by 50 μg/ml HA. Our results were in agreement with Radotra et al., where incorporation of HA into Matrigel increased the rate of invasion of glioma cells, by 70 percent. The increase was dose-dependent up to a concentration of 200 μg/cm² of HA, although the activation was reduced at higher concentrations (Radotra et al., 1997). It has been proposed that, in area of tumor invasion, HA is present at a high concentration and
degradation of the ECM takes place actively (Gayol et al., 2001 and Underhill, 1992). However, excess HA binding to CD44 can activate internalization and delivery to lysosome, leading to impaired cell invasion (Knudson et al., 2001).

KKU-M213 expressed at least two CD44 isoforms on the KKU-M213 cell membrane. In vitro invasiveness of KKU-M213 was activated by HA. Maximum activation was attained at 50 µg/ml HA, but this activation declined at higher concentrations of HA.

**Figure 1** Detection of CD44 mRNA in KKU-M213 by RT-PCR and agarose gel electrophoresis. The RT-PCR amplicons were separated on a 2% agarose gel electrophoresis. PCR amplicons of 569 bp and 965 bp were detected. The 569 bp amplicon corresponded to the size of the CD44s mRNA. The 965 bp amplicon is likely a variable isoform of CD44. β-actin was used an internal loading control.

**Figure 2** Expression of CD44 in KKU-M213 as determined by Immunofluorescence staining of live cells, 60× magnifications, Green color: FITC anti-mouse/human CD44, Red color: TOPROIII (nuclear staining)

**Figure 3** Activation of in vitro invasion of KKU-M213 by HA; bars, SE.
References