Expression of CD24 in cholangiocarcinoma cells is associated with disease progression and reduced patient survival

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Abstract. Cholangiocarcinoma is frequently found to invade local tissues and metastasize to distal organs. We investigated the expression of CD24 in cholangiocarcinoma samples and its prognostic significance. In addition, the cellular function of CD24 was studied in the RMCCA1 cholangiocarcinoma cell line. High CD24 expression significantly correlated with lymph node metastasis and positive surgical margins in cholangiocarcinoma patients. Univariate and multivariate analyses further demonstrated that CD24 expression was significantly associated with the overall survival of these patients (p=0.007 and p=0.040, respectively). For *in vitro* studies, the magnetic-activated cell sorting (MACS) system was used to isolate CD24$^+$ and CD24$^-$ cell populations from RMCCA1 cells. CD24$^+$ RMCCA1 cells had increased chemo-resistance, adhesion (p=0.004), motility (p<0.001), migration (p<0.001) and invasion (p<0.001) capabilities when compared to CD24$^-$ cells. The matrix metalloproteinase (MMP)-7 was significantly elevated in CD24$^+$ RMCCA1 cells (p=0.01). We found that inhibition of CD24 using siRNA silencing significantly decreased the invasive capacity of RMCCA1 cells. Both clinical and *in vitro* studies suggest that expression of CD24 is associated with cholangiocarcinoma disease progression. CD24 may thus serve as a new target for directed molecular therapy of cholangiocarcinoma.

Introduction

Cholangiocarcinoma is one of the most aggressive malignant tumors associated with local tissue invasion and a high rate of metastasis and is one of the most common causes of cancer death in Thailand (1). Three-year survival rates of 35-50% are achieved only in a subset of patients who have negative histological margins at the time of surgery (2-5). Palliative therapeutic approaches, including percutaneous and endoscopic biliary drainage, have generally been used for these patients because there is no effective chemotherapeutic treatment for cholangiocarcinoma. Therefore, identification of the molecules involved in cholangiocarcinoma cell progression is crucial for the development of novel drug treatments for this disease.

CD24 is a small, heavily glycosylated mucin-like glycosyl-phosphatidylinositol (GPI)-linked cell surface protein that serves as a ligand for P-selectin, an adhesion receptor on activated endothelial cells and platelets (6). CD24 can be detected in granulocytes, pre-B-cells and keratinocytes (7) and its expression in non-small cell lung cancer, ovarian cancer and hepatocellular carcinoma is associated with poor prognoses (8,9). However, only limited studies have been published on CD24 expression in cholangiocarcinoma (10,11) and there are no data on the mechanism of CD24 involvement in cholangiocarcinoma cell progression. In the present study, we investigated the clinicopathological significance of CD24 expression in human cholangiocarcinoma samples. We also determined the cellular function of CD24 in cholangiocarcinoma cells by isolating CD24$^+$ and CD24$^-$ cells from the RMCCA1 cholangiocarcinoma cell line and assessing cell proliferation, chemoresistance and invasiveness of CD24$^+$ versus CD24$^-$ cells.

Materials and methods

Human cholangiocarcinoma tissue samples. Cholangiocarcinoma tissue samples were obtained from cholangiocarcinoma patients who underwent a surgical resection at Rajavithi Hospital in Bangkok, Thailand from 2007-2009. The study was approved by the ethics committee of Rajavithi Hospital.

Immunohistochemical staining. Paraffin wax sections of cholangiocarcinoma specimens were dewaxed in xylene and transferred to alcohol. Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol, and the sections were boiled in 10 mM citrate buffer (pH 6.0) in a microwave oven

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(750 W) for antigen retrieval. Non-specific binding was blocked by incubation with 3% normal horse serum for 20 min. Sections were incubated overnight at 4˚C with a 1:1000 dilution of a mouse anti-CD24 monoclonal antibody. Biotinylated rabbit anti-mouse IgM (Dako, Glostrup, Denmark) was then added followed by an avidin-biotin-peroxidase conjugate (ABC Elite; Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. The immunohistochemical reaction was developed with freshly prepared reagents from a Histofine SAB-PO kit (Nichirei Inc., Tokyo, Japan). Sections were then visualized under high power magnification (x400) using an Olympus BH2 microscope (field width, 0.5 mm) and scored into one of the following three categories based on the percentage of positively stained cells: i) negative, < 5%, ii) low, 5-50% and iii) high, >50%.

Cell culture. The human cholangiocarcinoma cancer cell line, RMCCA1 (12), was grown in Ham's F-12 medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% FBS (fetal bovine serum) and 1% penicillin/streptomycin. In all experiments, cells were maintained at 37˚C in a humidified 5% CO_2 incubator.

Immunofluorescence study. To examine CD24 protein expression, cells were plated and grown on glass coverslips. After fixation with 4% paraformaldehyde for 15 min at room temperature, cells were treated with 4% bovine serum albumin. Cells were then stained with a mouse anti-CD24 antibody (mouse monoclonal IgG; Abcam Inc., Cambridge, MA) followed by an Alexa Fluor 594 goat anti-mouse IgG secondary antibody (1:200; Molecular Probes, Eugene, Oregon). The cells were stained with DAPI (4, 6-diamidino-2-phenylindole dihydrochloride) (Invitrogen) and viewed under a fluorescence microscope.

Isolation of $CD24^+$ and $CD24^-$ populations by magnetic cell sorting. For magnetic cell sorting, RMCCA1 cells were incubated with a FITC-conjugated anti-human CD24 antibody (Miltenyi Biotec, Auburn, CA), magnetically labeled with anti-FITC microbeads and then separated on a MACS MS column (Miltenyi Biotec) according to the manufacturer's instructions. The magnetic separation step was repeated twice to obtain high purity of the cell populations. The $CD24^+$ and $CD24^-$ sorted cells were then evaluated for the efficiency of the magnetic separation by flow cytometry.

Cell proliferation assay. For proliferation assays, cells were seeded into 96-well culture plates at a density of 1,000 cells per well. Cells were incubated for 0-9 days before applying the WST-1 cell proliferation assay reagent (Roche Diagnostics, Laval, Quebec, Canada) according to the recommendations of the manufacturer. The degree of cell proliferation was assessed by determining the A450 nm of the cell culture medium after addition of WST-1 for 2 h.

For cancer chemoresistance studies, 10,000 cells were treated with vehicle (normal medium) or 1-125 μg/ml cisplatin (Fresenius Kabi Oncology Ltd., Solan, India). Cells were then incubated for 48 h before applying WST-1. The number of viable cells after drug treatment was assessed using the WST-1 cell proliferation assay as described above. The drug concentration that resulted in a 50% decrease in relative absorbance was defined as the IC_{50}.

In vitro adhesion assay. Cells were seeded into 96-well culture plates coated with Matrigel at a density of 10,000 cells per well. Cells were allowed to adhere for 30 min. The non-adherent cells and media were then discarded, and the plates were washed with PBS. The number of adherent cells was assessed using WST-1.

Cell motility (wound-healing) assay. To assess cell motility, a wound-healing assay was performed followed by time-lapse live cell imaging. Wounds were generated by puncturing confluent cultures of cells with 20 μl pipette tips. These wounds were then covered with Matrigel to mimic cell communication with the microenvironment. Areas of wounds were marked and photographed every 15 min for 24 h using a Carl Zeiss Axio Observer Z1 inverted microscope (Carl Zeiss, Thornwood, NY). The distance of cell movement toward the wound space after 24 h was measured using live cell imaging software.

In vitro invasion assay. The invasiveness of cholangiocarcinoma cells was assayed in a 24-well Biocoat Matrigel invasion chamber (8 μm; Becton-Dickinson, Bioscience, Bedford, MA, USA). The upper chamber was seeded with 50,000 cells, and the lower chamber contained 1% FBS. After 24 h of incubation, the invading cells at the lower surface of the Matrigel-coated membrane were fixed with 25% methanol, stained with crystal violet (Sigma Chemical Co., St. Louis, MO) and counted in five random 400X power fields under a light microscope.

Migration assay. The migration assay was performed using 24-well transwell chambers (8 mm; Becton-Dickinson). A total of 20,000 cells were seeded into the transwell chamber and allowed to migrate for 24 h. The migrating cells at the lower surface of the transwell chamber were stained with crystal violet and counted in five random 400X power fields under a light microscope.

Inhibition of CD24 expression in RMCCA1 cells. Inhibition of CD24 expression was carried out using siRNA targeting CD24 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) according to the manufacturer's protocol. Briefly, $2x10^5$ cells were plated in 6-well tissue culture plates in antibiotic-free normal growth medium until the cells were 60-80% confluent. Cells were then incubated with a transfection reagent mixture (Ham's F-12, Lipofectamine (Invitrogen, Carlsbad, CA) and the $CD24^-$ directed oligonucleotides (20-80 pmols)) for 6 h at 37˚C. The transfection mixture was then replaced with normal growth medium, and the cells were incubated for 24 h. The medium was again replaced with fresh normal growth medium, and the cells were incubated for another 24 h before harvesting for experimental use. Block-iT Fluorescent Control (Invitrogen, Carlsbad, CA) was used to confirm the transfection efficiency.

Detection of matrix metalloproteinase (MMP)-9. Gelatin zymography was used to detect the expression of MMP-9 in $CD24^+$ and $CD24^-$ cells. Six million cells were plated in normal medium for 24 h of culture, and the medium was then replaced with serum-free medium. After another 24 h of culture, the conditioned medium was collected, mixed with non-reducing sample buffer (2% SDS, 10% glycerol, 62.5 mM
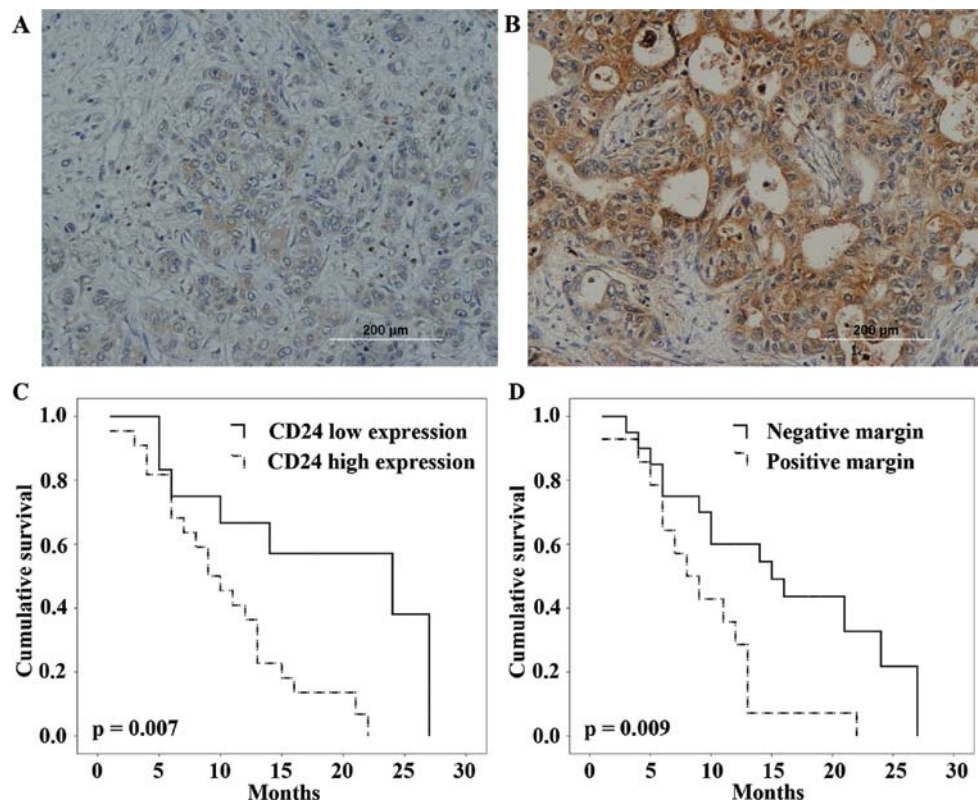
Figure 1. Representative immunohistochemical staining for CD24 in cholangiocarcinoma samples. (A) Cholangiocarcinoma cells with low expression of CD24. (B) Cholangiocarcinoma cells with high expression of CD24. Positive staining was observed in the cytoplasm of cholangiocarcinoma cells (magnification x200). (C) Survival curves of patients with high and low CD24 expression (p=0.007). (D) Survival curves of patients with positive and negative surgical margins (p=0.009).

Tris-Cl, pH 6.8, and 0.01% bromophenol blue) and separated on a 7.5% polyacrylamide gel containing 0.1% gelatin. After electrophoresis, the gel was soaked twice in 2.5% Triton X-100 in 50 mM Tris-Cl, pH 7.5, for 30 min and then incubated with 0.15 M NaCl, 10 mM $CaCl_2$ in 50 mM Tris-Cl, pH 7.5, for 18 h at 37˚C. The gel was then stained with 0.25% (w/v) Coomassie blue R250 and destained for 1 h.

Detection of matrix metalloproteinase (MMP)-7. The Human Total MMP-7 Immunoassay kit (R&D Systems, Minneapolis, MN) was used to detect the expression of MMP-7 in CD24+ and CD24- cells. Six million cells were plated in culture dishes. After 24 h of culture, the normal medium was replaced with serum-free medium, and the cells were cultured for another 24 h. The conditioned medium was collected and measured for the expression of MMP-7 following the manufacturer's protocol.

Statistical analysis. Statistical analysis of the association between clinicopathological data and the expression of CD24 was performed using the χ^2 test or Fisher's exact test. The Kaplan-Meier method was used to estimate survival as a function of time, and the survival differences were analyzed by log-rank test. The Cox regression model was used for multivariate analysis of prognostic factors. The cell proliferation and invasion assays were performed in triplicate, and each result was reported as the mean with standard deviation. Data were compared using the Student's t-test. A p<0.05 was considered statistically significant.

Results

Expression of CD24 in paraffin-embedded cholangiocarcinoma samples. Upon immunohistochemical analysis of non-cancer bile duct tissues, CD24 expression was not detected. In cancer tissues, specific CD24 signals were localized mainly in the cell membrane and cytoplasm of cholangiocarcinoma cells (Fig. 1A and B). We found that all cholangiocarcinoma samples (34/34) exhibited CD24 expression (>5% of cell staining). Applying the criteria for intensity of immunohistochemical staining for CD24, high expression (>50% of cell staining) of CD24 was noted in 64.7% (22/34) of cholangiocarcinoma samples. With respect to clinicopathological variables, we found that nodal metastasis (p=0.003) and a positive surgical margin (p=0.005) were more frequent in the CD24 high expression group compared with the CD24 low expression group (Table I). Univariate analysis revealed that the median survival time was 9 months in patients with high CD24 expression, whereas the survival time was 24 months in patients with low CD24 expression (p=0.007). We also found that the median survival time in patients with a negative surgical margin was significantly better than in patients with a positive surgical margin (15 months vs. 8 months; p=0.009) (Fig. 1C and D). In a multivariate Cox regression analysis, CD24 was indicated as an independent risk factor for survival (p=0.040; Table II).

In vitro study
Enrichment of CD24+ RMCCA1 cells. The results from the immunohistochemical staining indicated that high expression

Table I. Relationship between clinicopathological features and CD24 expression in cholangiocarcinoma samples.

Variable	Total (n=34)	CD24 expression		p-value
		Low	High	
Age				
<60	18	8	10	0.477
>60	16	4	12	
Sex				
Male	22	9	13	0.705
Female	12	3	9	
Tumor differentiation				
Well	17	9	8	0.071
Moderate/poor	17	3	14	
Node				
Negative	16	10	6	0.003[a]
Positive	18	2	16	
Distant metastasis				
Negative	24	11	13	0.061
Positive	10	1	9	
Surgical resection margin[b]				
R0	20	11	9	0.005[a]
R1, R2	14	1	13	

[a]Statistically significant. [b]Surgical resection margin: R0, negative resection margin; R1, microscopic positive resection margin; and R2, macroscopic positive resection margin.

of CD24 was associated with poor prognosis in cholangiocarcinoma patients. Therefore, we performed *in vitro* studies to identify the role of CD24 in cholangiocarcinoma cell progression. We isolated CD24$^+$ and CD24$^-$ cells from the RMCCA1 cholangiocarcinoma cell line using magnetic cell sorting technology. The percentage of CD24$^+$ cells in CD24-enriched cells (CD24$^+$) was 91.9%, and the percentage of CD24$^+$ cells in the CD24-depleted cells (CD24$^-$) was 11.5%; the percentage of CD24$^+$ cells in naïve RMCCA1 cells was 28.9% (Fig. 2A). As revealed by morphological studies, no differences in cell morphology were observed between CD24$^+$ and CD24$^-$ RMCCA1 cells (Fig. 2B).

Proliferation of CD24$^+$ and CD24$^-$ cholangiocarcinoma cells. CD24 expression has been shown to stimulate cancer cell proliferation. We therefore investigated the rate of cell proliferation in CD24$^+$ and CD24$^-$ RMCCA1 cells cultured for 0-9 days. We found that there was no statistically significant difference in cell proliferation between the CD24$^+$ and CD24$^-$ cell populations (Fig. 3A).

Chemoresistance of CD24$^+$ and CD24$^-$ RMCCA1 cells. Previous studies have suggested that CD24 expression is responsible for the lack of response to chemotherapy (10). Here, we investigated the response of CD24$^+$ and CD24$^-$ cells to cisplatin, an anticancer agent that is used to treat cholangiocarcinoma patients. The results demonstrated that CD24$^+$ cells had an IC$_{50}$ 1.7-fold higher (7.5 μg/ml) than the corresponding IC$_{50}$ value in CD24$^-$ cells (4.5 μg/ml) in response to cisplatin (Fig. 3B).

Cell adhesion assay in CD24$^+$ and CD24$^-$ RMCCA1 cells. Previous work has demonstrated that CD24 enhances cell adhesion (6). Therefore, we investigated the role of CD24 in cell adhesion in CD24$^+$ and CD24$^-$ RMCCA1 cells. Our data demonstrated that CD24$^+$ cells adhered to Matrigel coated plates significantly better than CD24$^-$ cells (p=0.004). However, cell adhesion to non-coated plates was not significantly different between CD24$^+$ and CD24$^-$ cells (Fig. 4A). This finding suggests that CD24 enhances cell adhesion by allowing cells to adhere to extracellular matrix proteins.

Cell motility assay in CD24$^+$ and CD24$^-$ RMCCA1 cells. A previous study reported that CD24$^+$ cells are more motile than CD24$^-$ cells (13). In our work, time-lapse live cell imaging was performed to investigate cell migrating across a wound gap. The motility of CD24$^+$ cells to cover the wound was significantly greater than that of the CD24$^-$ cells (p<0.001) (Fig. 4B).

Table II. Survival analysis based on clinicopathological parameters using Cox's multivariate model.

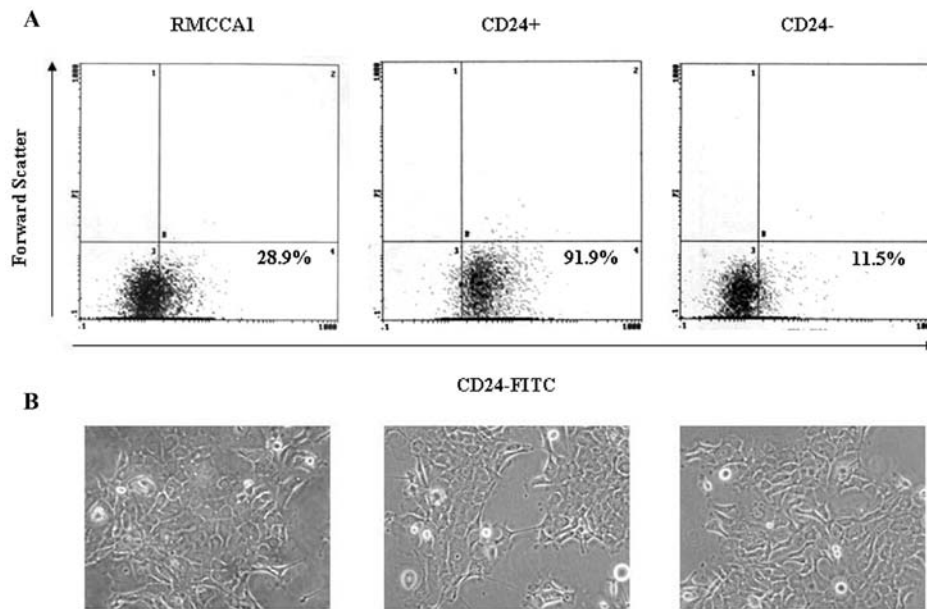| Variables	p-value	Hazard ratio	95% confidence interval	
			Lower limit	Upper limit
Tumor differentiation	0.26	0.58	0.22	1.51
Lymph node metastasis	0.49	0.66	0.19	2.02
Distant metastasis	0.45	0.64	0.20	2.06
Surgical resection status	0.11	2.66	0.80	8.78
CD24 expression	0.04[a]	4.31	1.06	17.48

[a]Statistically significant.

Figure 2. Analysis of purity of sorted populations. CD24$^+$ and CD24$^-$ cell fractions were separated from RMCCA1 cells using MACS. Sorted CD24$^+$ and CD24$^-$ cell subsets were analyzed by flow cytometry to evaluate the efficiency of sorting. (A) Percentage of CD24$^+$ cells in RMCCA1, the enriched (CD24$^+$) and depleted (CD24$^-$) fractions. (B) Cell morphology of CD24$^+$ and CD24$^-$ fractions.
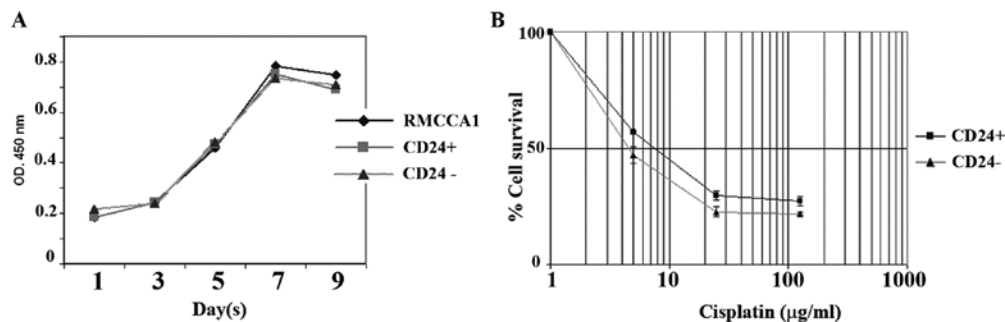

Figure 3. (A) MACS-sorted CD24$^+$ and CD24$^-$ cells exhibit a similar degree of cell proliferation. Cells were isolated by MACS and subjected to *in vitro* assays for cell proliferation. Cell growth kinetics in normal culture over time in RMCCA1 cells (◆); in CD24$^+$ cells (■); and in CD24$^-$ cells (▲) are shown. (B) Chemoresistance of CD24$^+$ and CD24$^-$ cells to cisplatin. The IC$_{50}$ represents the drug concentration that resulted in a 50% decrease in cell survival.
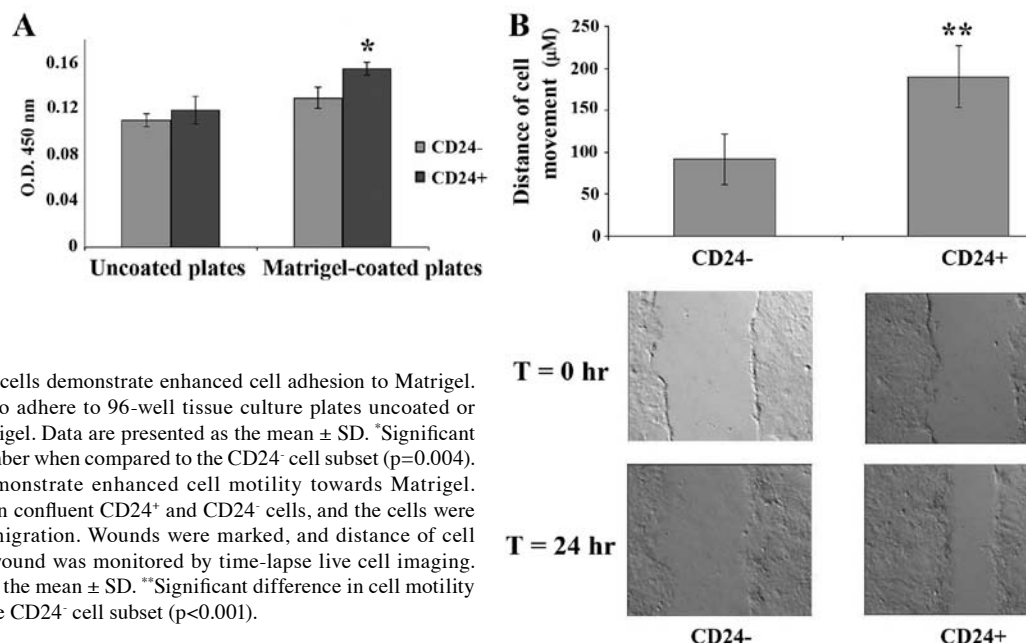

Figure 4. (A) CD24$^+$ cells demonstrate enhanced cell adhesion to Matrigel. Cells were allowed to adhere to 96-well tissue culture plates uncoated or pre-coated with Matrigel. Data are presented as the mean ± SD. *Significant difference in cell number when compared to the CD24$^-$ cell subset (p=0.004). (B) CD24$^+$ cells demonstrate enhanced cell motility towards Matrigel. Wounds were made in confluent CD24$^+$ and CD24$^-$ cells, and the cells were allowed to heal by migration. Wounds were marked, and distance of cell movement into the wound was monitored by time-lapse live cell imaging. Data are presented as the mean ± SD. **Significant difference in cell motility when compared to the CD24$^-$ cell subset (p<0.001).
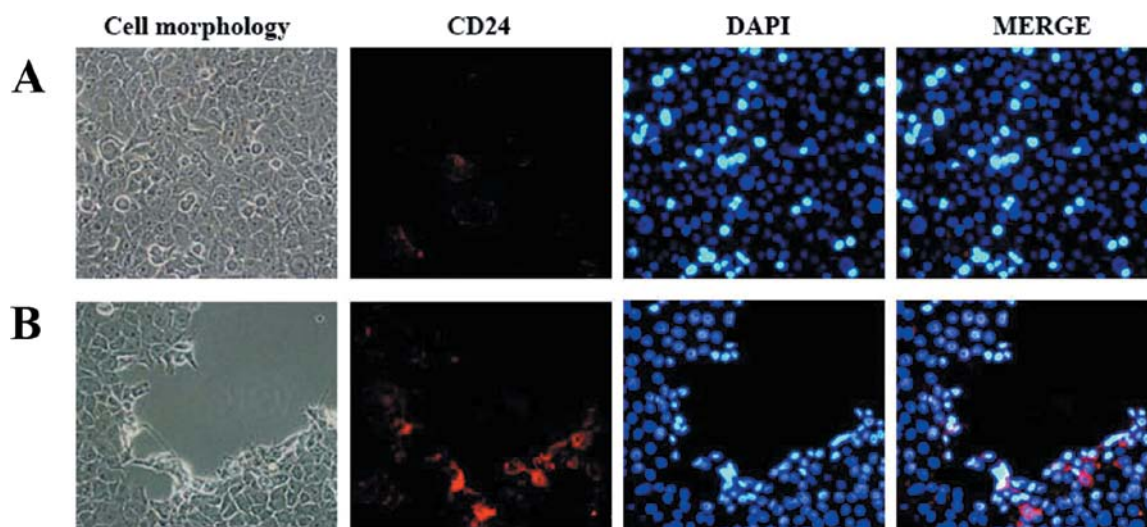
Figure 5. Wounds were created in confluent RMCCA1 cells, and immunofluorescence staining was performed to compare (A) CD24 expression in cells distant from and (B) cells at the wound edge. Cells at the wound edge expressed high levels of CD24 compared to the cells distant from the wound.
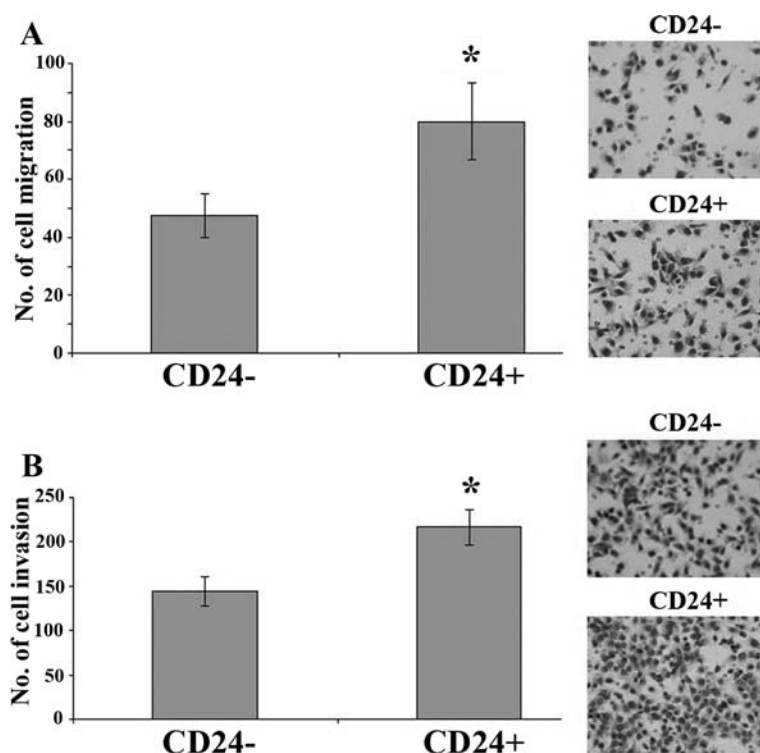

Figure 6. (A) CD24⁺ cells demonstrate enhanced cell migration and (B) cell invasion *in vitro*. The migration and invasion capabilities of CD24⁻ and CD24⁺ cells isolated from the RMCCA1 cell line were assessed. Transwells were uncoated (migration assays) or pre-coated with Matrigel (invasion assays), and cells were allowed to migrate or invade for 24 h and counted. Data are presented as the mean ± SD. *Significant difference in cell number when compared to the CD24⁻ cell subset (p<0.001).

We also performed a wound-healing assay in naïve RMCCA1 cells. Immunofluorescence staining with an anti-CD24 antibody demonstrated that the cells at the wound edge expressed higher levels of CD24 than the cells distant from the sight of the wound (Fig. 5).

Cell migration and invasion assay in CD24⁺ and CD24⁻ RMCCA1 cells. Previous studies have shown that CD24 expression is associated with tumor aggressiveness, as demonstrated by increased metastasis and invasiveness (13,14). In the present study, transwell migration and invasion assays were performed to assess the metastatic potential of the cell populations. The CD24⁺ cell subset showed a significantly greater ability to migrate towards serum (Fig. 6A) and invade through Matrigel (Fig. 6B) than the CD24⁻ cell subset (p<0.001).

Detection of MMP-9 and MMP-7 in the conditioned medium of CD24⁺ and CD24⁻ RMCCA1 cells. Matrix metalloproteinases
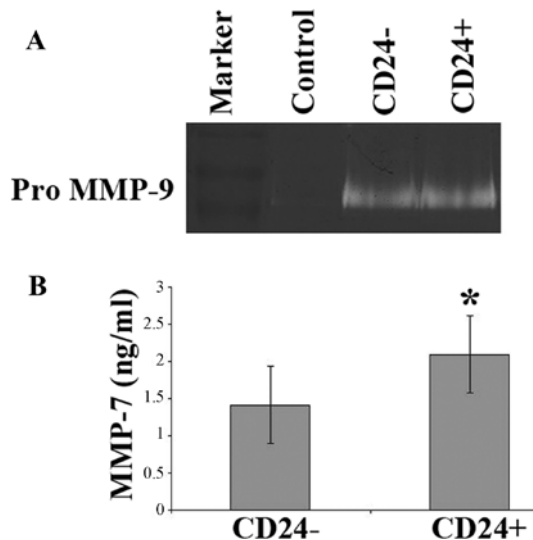
Figure 7. (A) MMP-9 activity in CD24^{+} and CD24^{-} cell populations; medium with 1% FBS (positive control) and conditioned medium from CD24^{+} and CD24^{-} cell populations were subjected to gelatin zymography. Matrix metalloproteinase activity is shown as a clear band on the gel. (B) MMP-7 levels in CD24^{+} and CD24^{-} cell populations. The secreted level of MMP-7 in the conditioned medium collected from both CD24^{+} and CD24^{-} cell populations was assessed by the Human Total MMP-7 ELISA kit. Data are presented as the mean ± SD. *Significant difference in cell number when compared to the CD24^{-} cell subset (p=0.01).

(MMPs) have long been associated with cancer-cell invasion and metastasis via the cleavage of basement membrane and extracellular matrix proteins to allow for cells to invade tissues and spread throughout the body (15,16). Here, we investigated the expression of MMP-9 by harvesting conditioned medium from both CD24^{+} and CD24^{-} cell populations and performing gelatin zymography. We found that the conditioned medium from both cell populations expressed similar levels of MMP-9 as observed by the similar intensity of the clear band at ~92 kDa, which corresponds to the known size of pro-MMP-9 (Fig. 7A).

We also examined the secreted level of MMP-7 in the conditioned medium of both cell populations by enzyme-linked immunosorbent assay (ELISA). The results showed that MMP-7 levels were significantly higher in the conditioned medium of CD24^{+} cells than in the conditioned medium of CD24^{-} cells (p=0.01) (Fig. 7B).

Inhibition of CD24 expression decreased cell invasion. We further sought to confirm the correlation between CD24 expression and cell invasion by silencing CD24 expression in RMCCA1 cells. As demonstrated by immunofluorescence, RMCCA1 cells that were treated with CD24 siRNA showed a reduced CD24 signal when compared with the cells treated with control siRNA (Fig. 8A). The results of the invasion assay showed that the cells
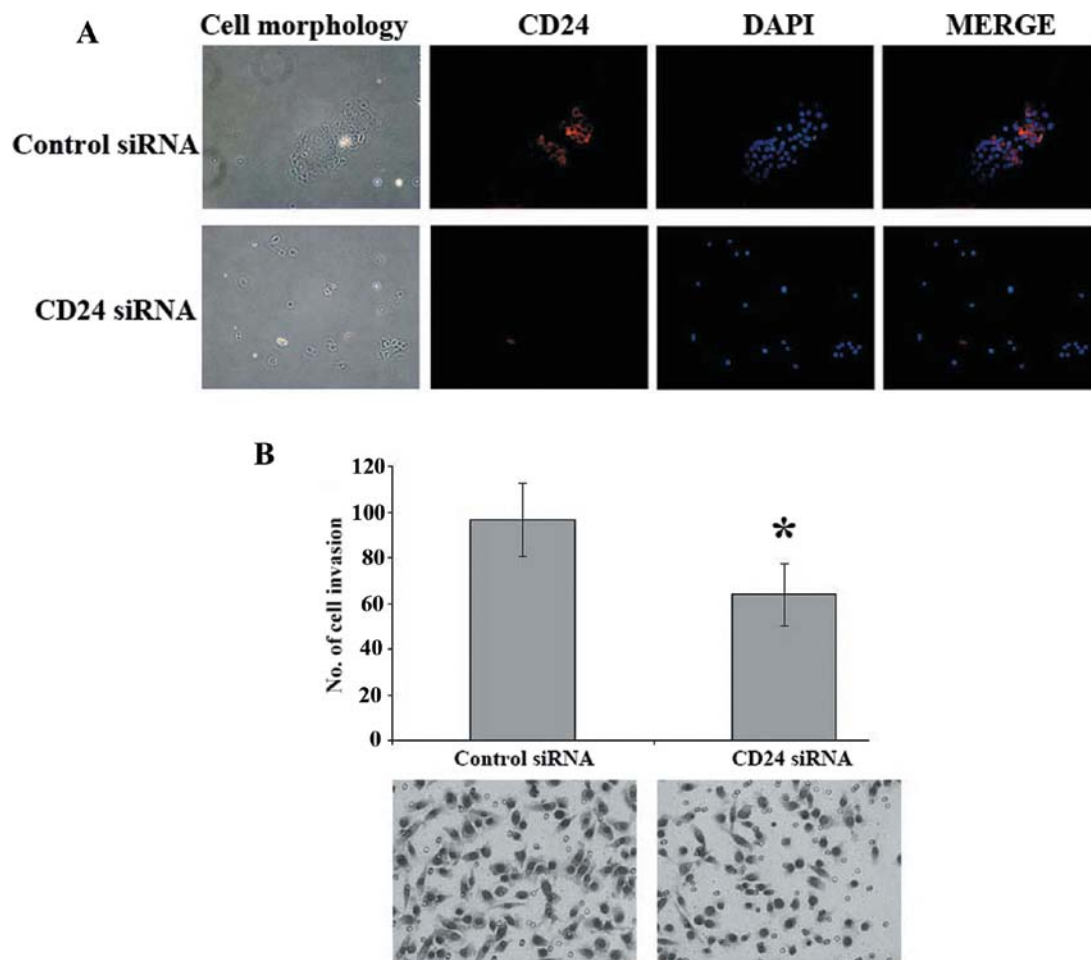

Figure 8. Inhibition of CD24 expression results in decreased cell invasion. (A) Immunofluorescence staining in RMCCA1 cells and CD24 siRNA-treated RMCCA1 cells using an anti-CD24 antibody. (B) Cell invasion capabilities of RMCCA1 cells treated with control or CD24 siRNA. Data are presented as the mean ± SD. *Significant difference in cell number when compared to the CD24^{-} cell subset (p<0.001).

that were treated with CD24 siRNA had a significantly diminished capacity to invade through the Matrigel when compared with the cells treated with control siRNA (p<0.001) (Fig. 8B).

Discussion

In this study, we demonstrated that 64.7% of the cholangiocarcinoma samples exhibited high expression of CD24. Moreover, we showed that high expression of CD24 correlated with lymph node metastasis and overall survival of the cholangiocarcinoma patients. These findings are consistent with a previous study, which demonstrated that cholangiocarcinomas highly expressing CD24 tended to be associated with higher incidences of metastasis and worse prognoses (10,11). In addition, many studies have shown that CD24 expression correlates with the aggressiveness of other types of cancer, including pancreatic cancer (17) and hepatocellular carcinoma (18). Taken together, our data indicate that CD24 is involved in the progression and malignant behavior of cholangiocarcinoma.

To understand the role of CD24 in cholangiocarcinoma cells, we compared the cell proliferation, chemoresistance and invasion potential of isolated CD24+ and CD24- RMCCA1 cholangiocarcinoma cell populations. We found no difference in cell proliferation between CD24+ and CD24- RMCCA1 cells. However, the chemoresistance assay demonstrated that CD24+ RMCCA1 cells exhibited more resistance to cisplatin than CD24- RMCCA1 cells. This result is also consistent with recent evidence showing that the mechanism of drug resistance in colon and pancreatic cancer cells (19) is through the expression of CD24. Our studies therefore indicate that expression of CD24 may be the key mechanism governing the chemoresistant phenotype of cholangiocarcinoma. Further studies are required to identify the role of CD24 in cholangiocarcinoma patients receiving chemotherapy.

Our results are consistent with previous work demonstrating that downregulation of CD24 in colorectal and pancreatic cancer by RNA interference or by using anti-CD24 monoclonal antibodies significantly inhibit cell invasion (19). We demonstrated that inhibiting CD24 by siRNA reduced the invasive capacity of RMCCA1 cells, indicating that CD24 plays an important role in cholangiocarcinoma progression via its potential to promote invasion. The requirements for tumor cell invasion consist of the following: i) adhesion of tumor cells to the extracellular matrix (ECM), ii) migration of tumor cells through the ECM and iii) degradation of the ECM by cellular proteases (20). The ability of CD24+ cells to invade has generally been reported to be due to the adhesion between CD24 and extracellular molecules. A previous study demonstrated that the CD24high MCF-7 cells were more adhesive than the CD24low cells (21). In our study, we also observed that CD24+ cells had a greater ability to adhere to Matrigel than CD24- cells. However, there was no significant difference between CD24+ and CD24- cells to adhere to non-coated culture plates. It is likely that the increased adhesive ability of CD24+ cells was due to the binding of CD24 with the extracellular molecules in the Matrigel. Taken together, these results suggest that CD24 enhances the adhesiveness of cholangiocarcinoma cells.

We investigated the capability of cells to migrate by using a wound-healing assay. After a wound is created in cultured confluent cells, cells at the edge of the wound will migrate across the wound gap. We found that the motility of CD24+ cells was higher than that of CD24- cells. This finding is consistent with a previous study in which forced expression of CD24 in HCT116 human colon cancer cells significantly increased cell migration in wound-healing assays (22). We also performed a wound-healing assay in naïve RMCCA1 cells and found that RMCCA1 cells at the wound edge expressed higher levels of CD24, suggesting that CD24- cells can give rise to CD24+ cells that have a greater ability to migrate. These data indicate that cholangiocarcinoma cells can change their protein expression in order to acquire the capacity to invade.

We further examined the expression of proteases in CD24+ and CD24- cells. We demonstrated for the first time that MMP-7 was highly expressed in CD24+ cholangiocarcinoma cells. Previous studies have demonstrated that MMP-7 plays a key role in the mechanism of cancer invasion via proteolytic cleavage of the extracellular matrix tissues (22). MMP-7 has also been shown to activate other MMPs, such as pro-MMP-2 and pro-MMP-9, and to inhibit E-cadherin function by inducing ectodomain shedding of E-cadherin (23,24). The results of several recent studies have indicated that MMP-7 is overexpressed in a variety of epithelial cancers, including esophagus, colon, pancreas, and cholangiocarcinoma, and is associated with cancer progression (25-28). Thus, the invasive capacity of CD24+ cells not only depends on their adherent capabilities but also on increased MMP-7 expression.

In conclusion, CD24 is highly expressed in cholangiocarcinoma, and it significantly correlates with lymph node metastasis. CD24 should therefore be used as a potential prognostic factor and target for treatment of cholangiocarcinoma.

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