Matrigel induces L-plastin expression and promotes L-plastin-dependent invasion in human cholangiocarcinoma cells

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Abstract. The function of the extracellular matrix (ECM) in the tumor microenvironment is not limited to forming a barrier against tumor invasion. As demonstrated in pathological specimens, cholangiocarcinoma samples exhibit an enrichment of the ECM surrounding the tumor cells. In this study, we examined involvement of the ECM in the regulation of the invasiveness of cholangiocarcinoma cells. The RMCCA1 cholangiocarcinoma cell line was cultured in culture plates either with or without a coating of reconstituted ECM basement membrane preparation (BD Matrigel matrix). In vitro invasion assays were then performed. In addition, the protein expression profile of the cell line was examined using two-dimensional gel electrophoresis and liquid chromatography-tandem mass spectrometry. The proteins expressed and their functional associations with cancer progression were determined. Culturing the RMCCA1 cell line in the BD Matrigel matrix induced cell invasion. Numerous proteins were induced by culturing the RMCCA1 cells in the matrix gel. The expression of L-plastin, an actin-binding protein, was significantly upregulated. The knockdown of L-plastin expression by siRNA silencing significantly suppressed the cellular response to matrix gel-stimulated cancer cell invasion. The ECM promotes the invasiveness of cholangiocarcinoma cells by upregulating L-plastin. These findings suggest the potential exploitation of this mechanism as a means of inhibiting the invasiveness of cholangiocarcinoma cells.

Introduction

Cholangiocarcinoma, an aggressive malignant tumor that develops from the bile duct epithelium, is associated with local invasiveness and a high rate of metastasis (1,2). The worldwide incidence and mortality rates associated with cholangiocarcinoma have risen over the past three decades. In Thailand, the annual incidence of cholangiocarcinoma is 87 per 100,000 inhabitants (3). In the United States, the most commonly recognized risk factor for cholangiocarcinoma is primary sclerosing cholangitis (4). However, in Southeast Asia and particularly in Thailand, infection with hepatobililiary flukes (Opisthorchis viverrini) is the most common risk factor for cholangiocarcinoma (5). Therapeutic options for cholangiocarcinoma patients are limited, as this type of cancer responds poorly to chemotherapy and radiation therapy. Surgery is thus the only potentially effective treatment for cholangiocarcinoma. However, typical five-year survival rates of 32-50% are achieved only by a small number of patients with negative histological margins at the time of surgery (6-8). Therefore, the understanding of the mechanisms involved in cancer cell invasion and metastasis may be useful in developing new therapeutic options for cholangiocarcinoma patients.

The function of the extracellular matrix (ECM) in the tumor microenvironment is not limited to forming a barrier against tumor invasion. Previous studies have indicated that interactions between cancer cells and the ECM play an important role in cancer progression. The molecular components of the ECM, such as fibronectin, laminin, collagen and heparin sulfate proteoglycans, communicate with cancer cells and modulate a variety of cellular functions required for cancer cells to exhibit invasive and metastatic properties (9-11). Numerous results from pathological studies have indicated that cholangiocarcinoma cells are surrounded by a dense sheath of connective tissue that contains the ECM (12-14). However, there have been no studies to date regarding the definitive role that the ECM plays in cholangiocarcinoma cell invasion. Therefore, we aimed to investigate the involvement of the ECM in cholangiocarcinoma cell invasion.
Materials and methods

Cell cultures. The RMCCA1 human cholangiocarcinoma cell line, originally derived from a cholangiocarcinoma patient (15), was grown in Ham’s F12 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) at 37°C in a 5% CO₂ humidified atmosphere.

Cell invasion assay. To study the mechanism of cancer cell invasion in vitro, RMCCA1 cells were cultured in BD Matrigel matrix (BD Biosciences, Bedford, MA, USA) for 0-24 h. Next, cancer cells were seeded into porous cell culture insert cups (BD Biosciences) each containing a layer of matrix gel. The number of cancer cells that invaded through the basement membrane within 24 h was assessed by staining the cells with crystal violet (Sigma-Aldrich, St. Louis, MO, USA) (16).

Two-dimensional (2D) gel electrophoresis. 2D gel electrophoresis was performed for the analysis of proteins extracted from cholangiocarcinoma cells cultured in uncoated and 24-h matrix gel-coated plates. Each electrophoresis gel contained three pooled samples from the cell culture plates. Six gels were prepared in biological triplicates from the uncoated and matrix gel-coated plates. Protein samples (500 µg) were applied to 18-cm immobilized pH gradient (IPG) gel strips (pH 3-10; GE Healthcare, Uppsala, Sweden) by cup loading near the anodic ends of the strips. Isoelectric focusing (IEF) was performed using an Ettan IPGphor Manifold on an Ettan IPGphor isoelectric focusing unit (GE Healthcare) for 32,000 Vh at 20°C. Following IEF, each gel strip was equilibrated with equilibration buffer. The IPG strips were then loaded and run on 12.5% acrylamide gels (GE Healthcare) using the Ettan DALTsix electrophoresis system (GE Healthcare). The run was stopped after the bromophenol blue dye front had run off the bottom of the gels. The gels were then stained with colloidal Coomassie Blue (GE Healthcare).

2D image analysis. The proteins were visualized using an ImageScanner (GE Healthcare). The gel images were analyzed to determine differential protein expression profiles using ImageMaster 2D Platinum software (GE Healthcare). Student’s t-test was used for statistical analysis and P<0.05 was considered to indicate a statistically significant difference.

Protein identification by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

In-gel digestion. LC-MS/MS was performed by the Proteomics Laboratory, Genome Institute, National Science and Technology Development Agency (Pathumthani, Thailand). Following 2D analysis, an in-gel digestion was performed. Briefly, after the protein spots were excised, the gel plugs were dehydrated with 100% acetonitrile (ACN), reduced with 10 mM DTT in 10 mM ammonium bicarbonate at room temperature for 1 h and alkylated at room temperature for 1 h in the dark in the presence of 100 mM iodoacetamide in 10 mM ammonium bicarbonate. Following alkylation, the gel pieces were dehydrated twice with 100% ACN for 5 min. For the in-gel digestion of the proteins, 10 µl trypsin solution (20 ng/µl trypsin in 50% ACN/10 mM ammonium bicarbonate) was added to the gels followed by incubation at room temperature for 20 min. Next, 20 µl 30% ACN was added to keep the gels immersed throughout digestion. The gels were incubated at 37°C overnight. To extract the peptide digestion products, 30 µl 50% ACN in 0.1% formic acid was added to the gels, which were then incubated at room temperature for 10 min in a shaker. The extracted peptides were collected and pooled in a new tube. The pooled extracted peptides were dried by vacuum centrifugation at 2,500 x g for 10 min and stored at -80°C until further mass spectrometric analysis.

LC-MS/MS analysis. The LC-MS/MS analysis of the digested peptide mixtures was performed using a Waters SYNAPT™ HDMS™ system (Waters, Milford, MA, USA). The 1D-nanoLC was performed with a Waters nanoACQUITY UPLC system (Waters). Tryptic digests (4 µl) were injected onto an reversed-phase analytical column (20 cm x 75 µm) packed with 1.7-µm ethylene bridged hybrid C18 material (Waters). The peptides were eluted with a linear gradient of 2-40% acetonitrile developed over 30 min at a flow rate of 1000 nl/min. This elution was followed by a 10-min 80% acetonitrile treatment to clean the column before using 2% acetonitrile for the next sample. The effluent samples were electrosprayed into a mass spectrometer (SYNAPT HDMS system) for MS/MS analysis of the peptides, and spectral data were generated for further protein identification by matching against hits in a database search.

Mass lists in the form of Mascot generic files were created and used as the inputs for the Mascot MS/MS Ion web-based search functionality at the National Center for Biotechnology Information non-redundant database (www.matrixscience.com). The default search parameters were applied as follows: Enzyme, trypsin; taxonomy, Homo sapiens (human); maximum missed cleavages, 1; fixed modifications, carbamylomethyl (C); variable modifications, oxidation (M); peptide tolerance, ±1.2 Da; MS/MS tolerance, ±0.6 Da; peptide charge, 1+, 2+ and 3+; and instrument, ESI-QUAD-TOF.

Western blot analysis. Protein extracts isolated from the cells cultured in the uncoated and 24-h matrix gel-coated plates were separated by 12% SDS-PAGE and then transferred onto a nitrocellulose membrane (GE Healthcare). The membrane was subsequently incubated with monoclonal antibodies against L-plastin (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β-actin (1:500; Cell Signaling Technology, Danvers, MA, USA). Horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) and anti-rabbit IgG at 1:5,000 dilutions were used as secondary antibodies (GE Healthcare). The blots were visualized using an ECL Plus detection kit and Hyperfilm ECL (GE Healthcare). The western blot results were quantified using densitometer and image analysis software (ImageScanner III and ImageQuant TL; GE Healthcare, Uppsala, Sweden).

Inhibition of L-plastin expression using transient siRNA transfection. L-plastin siRNA (Santa Cruz Biotechnology) was used to knock down L-plastin gene expression. A fluorescein-labeled, double-stranded RNA duplex (BLOCK-it™ Fluorescent Oligo; Invitrogen, Melville, NY, USA) was designed as a control. The siRNA molecules were diluted in Opti-MEM® I Medium without serum (Gibco) and mixed
Culturing cholangiocarcinoma cells in matrix gel increases their invasiveness. RMCCA1 cholangiocarcinoma cells were incubated in matrix gel for 0-24 h, and invasion assays were then performed. The results showed that a significantly higher number of cholangiocarcinoma cells that were cultured in matrix gel invaded through the insertion cup compared with that observed with the cells that were cultured on uncoated plates (P<0.001; Fig. 1).

Proteomic study of cholangiocarcinoma cells cultured in matrix gel. To investigate the proteins potentially involved in cholangiocarcinoma cell invasion, cholangiocarcinoma cells were cultured in plates coated with or without matrix gel. Next, 2D gel electrophoresis using pH 3-10 Linear IPG strips was performed to identify the protein expression profiles of these cells. Approximately 800 protein spots were detected by colloidal Coomassie staining. Quantitative intensity and statistical analyses identified 129 protein spots with significantly altered expression levels in matrix gel culture compared with the uncoated culture system. Of these 129 proteins, 60 proteins exhibited greater than two-fold upregulation in the presence of matrix gel.

Results

Figure 1. RMCCA1 cholangiocarcinoma cell invasion assays. (A) Box plots comparing the number of cholangiocarcinoma cell invasion events in cells cultured in matrix gel and controls (P<0.001 by analysis of variance, compared with the control). (B) Micrographs of cholangiocarcinoma cell invasion (cholangiocarcinoma cells were cultured in matrix gel for 0, 6, 12, 18 or 24 h before the invasion assays were performed). The scale bar indicates 100μm (magnification, x10).

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Table I. A summary of upregulated proteins expressed in cholangiocarcinoma cells cultured in matrix gel, as identified by Q-TOF MS and MS/MS analyses.

| Functional category and protein name | GI number | Mr (kDa) | pI | Score | Coverage % | Ratio | Gene ID | Cellular component |
|-------------------------------------|-----------|----------|----|-------|------------|-------|---------|-------------------|
| Actin-binding protein               |           |          |    |       |            |       |         |                   |
| L-plastin                           | 62087548  | 56,196   | 5.21| 52    | 6          | 4.6   | LCP1, PLS2, VIL2, EZR | Cytoplasm, cell membrane, cytoskeleton |
| Cytofilin 2 (Ezrin)                 | 340217    | 68,233   | 5.80| 335   | 13         | 3.9   | ACTR3, EZR | Cytoplas, cell membrane, cytoskeleton |
| ARP3 actin-related protein 3 homolog| 5031573   | 47,797   | 5.61| 150   | 6          | 3.5   | ACTN4, EZR | Cytoplasm, nucleus |
| α-actinin-4                         | 2804273   | 102,661  | 5.27| 72    | 2          | 3.0   | CAP1, ACTN4 | Cell membrane |
| Adenylyl cyclase-1 associated protein| 116241280| 52,222   | 8.27| 54    | 10         | 2.6   | CFL1, ACTN4 | Cytoplasm, cytoskeleton |
| Fascin                              | 4507115   | 55,123   | 6.84| 160   | 19         | 2.5   | FSCN1, ACTN4 | Cytoplasm, cytoskeleton |
| Cofilin-1                           | 5031635   | 18,719   | 8.22| 129   | 17         | 2.3   | CFL1, ACTN4 | Cytoplasm, nucleus, cell membrane, cytoskeleton |
| Energy metabolism                   |           |          |    |       |            |       |         |                   |
| Pyruvate Kinase (Pkm2)              | 6746392   | 60,277   | 8.22| 166   | 20         | 7.3   | PKM2, PGK1, PGK | Cytoplasm, nucleus |
| Phosphoglycerate kinase 1           | 4505763   | 44,985   | 8.30| 439   | 20         | 6.3   | PGK1, PGKA | Cytoplasm |
| Aldolase A                          | 28614     | 39,706   | 8.34| 267   | 11         | 3.5   | ALDOA, ALDA | Cytoplasm, nucleus |
| α-enolase (phosphopyruvatehydratase)| 693933    | 47,421   | 7.01| 148   | 22         | 2.4   | ENO1, ALDOA | Cytoplasm, nucleus |
| L-lactate dehydrogenase A chain     | 126047    | 36,950   | 8.44| 85    | 18         | 2.3   | LDHA, PIG19 | Cytoplasm |
| ATP synthase, H+ transporting, mitochondrial F1 complex | 4757810  | 59,828   | 9.16| 182   | 17         | 13.5  | ATP5A1, ATP5F1 | Mitochondrion, inner membrane |
| Dihydrolipoamidesuccinyl transferase| 643589    | 48,896   | 8.90| 165   | 7          | 3.8   | DLST, ATP5A | Mitochondrion |
| Citrate synthase                    | 33337556  | 51,942   | 8.45| 169   | 5          | 3.4   | CS, ATP5A | Mitochondrion |
| Fumaratehydratase, mitochondrial    | 182794    | 50,524   | 7.23| 305   | 11         | 2.7   | FH, ATP5A | Mitochondrion, cytoplasm |
| Glutamat dehydrogenase              | 4582581   | 61,701   | 7.66| 197   | 16         | 3.9   | GLUD1, ATP5A | Mitochondrion |
| Dihydrolipoamide dehydrogenase      | 8375380   | 50,656   | 6.50| 236   | 9          | 2.2   | DLD, ATP5A | Mitochondrion |
| acyl-Coenzyme A dehydrogenase       | 76496475  | 68,414   | 8.76| 233   | 10         | 2.1   | ACADVL, ATP5A | Mitochondrion, inner membrane |
| Transketolase                        | 37267     | 68,435   | 7.90| 102   | 16         | 2.8   | TKT, ATP5A | Cytoplasm, nucleus |
| Molecular chaperone                  |           |          |    |       |            |       |         |                   |
| Tumor rejection antigen 1, Endoplasmin| 74755280 | 92,282   | 4.77| 89    | 4          | 11.9  | GRP94, HSP90B1 | Endoplasmic reticulum, cytoskeleton |
| T-complex protein 1 subunit γ        | 14124984  | 60,934   | 6.10| 157   | 7          | 6.3   | CCT3, CCTG | Cytoplasm |
| Heat shock protein HSP 90-α          | 154146191 | 85,006   | 4.94| 163   | 5          | 5.2   | HSP90AA1 | Mitochondrion |
| Heat shock protein HSP 90-β          | 119602173 | 57,868   | 4.92| 59    | 2          | 2.3   | HSP90AB1 | Mitochondrion |
| Stress-induced-phosphoprotein 1 (Hsp70/Hsp90-organizing protein) | 58031811 | 63,227   | 4.60| 145   | 19         | 3.8   | STIP1, HSP90B1 | Cytoplasm, nucleus |
| 60 kDa heat shock protein            | 77702086  | 61,346   | 5.70| 580   | 16         | 5.0   | HSPD1, HSP60 | Mitochondrion |
| Heat shock protein                   | 386785    | 70,110   | 5.42| 404   | 10         | 4.4   | HSPA1L, HSP90B1 | Mitochondrion |
| Heat shock 70 kDa protein 8          | 5729877   | 71,082   | 5.37| 203   | 13         | 3.8   | HSPA8, HSP90B1 | Mitochondrion |
| Stress-70 protein, mitochondrial    | 2164428   | 73,920   | 5.87| 70    | 6          | 3.7   | HSPA9, HSP90B1 | Mitochondrion |
| 78 kDa glucose-regulated protein    | 3867587   | 72,185   | 5.03| 251   | 7          | 4.1   | GRP78, HSPA5 | Endoplasmic reticulum |
| T-complex polypeptide 1              | 36796     | 60,669   | 6.03| 197   | 9          | 4.0   | TCP1, HSP90B1 | Cytoplasm, nucleus |
| Nucleophosmin                        | 15214852  | 32,760   | 4.64| 198   | 12         | 3.3   | NPM1, NPM | Mitochondrion, cytoskeleton |
| (nucleolarphosphoprotein B23, numatrin) |           |          |    |       |            |       |         |                   |
| Calretilcin                          | 4757900   | 48,283   | 4.29| 109   | 12         | 2.3   | CALR, HSPA60 | Cytoplasm, endoplasmic reticulum, extracellular matrix, secreted |
| Structural molecule                  |           |          |    |       |            |       |         |                   |
| Tubulin, β 2                         | 5174735   | 50,255   | 4.79| 282   | 14         | 3.8   | TUBB2C, TUBB4B | Cytoplasm, cytoskeleton, microtubule |
| α-tubulin                            | 37492     | 50,810   | 5.02| 123   | 13         | 2.6   | TUBA4A, TUBA1 | Cytoplasm, cytoskeleton, microtubule |
| Cytoskeleton function                |           |          |    |       |            |       |         |                   |
| Keratin, type I cytoskeletal 17      | 4557701   | 48,361   | 4.97| 303   | 28         | 3.7   | KRT17, TUBB2C | Cytoplasm, intermediate filament, keratin |

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results, these proteins are involved in energy metabolism, molecular chaperoning, cytoskeleton functions, actin binding, translation, transcription regulation, calcium ion binding, cell structure and signal transduction (Fig. 2A). Contact with the ECM and the remodeling of the actin cytoskeleton can drive cancer cell motility and promote invasion. L-plastin is one of the actin-binding proteins that exhibited a high level of protein expression in cholangiocarcinoma cells cultured in matrix gel. We performed a western blot analysis to confirm the results of the proteomic study. The results showed that a high level of

| Functional category and protein name | GI number | Mr  | pI  | Score | Coverage % | Ratio | Gene ID | Cellular component |
|-------------------------------------|-----------|-----|-----|-------|------------|-------|---------|-------------------|
| Keratin, type I cytoskeletal 18     | 30311     | 47,305 | 5.27 | 558   | 28         | 3.5   | KRT18, PIG46 CYK18 | Cytoplasm, intermediate filament, keratin |
| Keratin, type I cytoskeletal 19     | 24234699  | 44,079 | 5.04 | 455   | 44         | 2.1   | KRT19            | Intermediate filament, keratin |
| Keratin, type II cytoskeletal 6A    | 5031839   | 60,293 | 8.09 | 248   | 29         | 5.8   | KRT6A            | Intermediate filament, keratin |
| Keratin, type II cytoskeletal 2 epidermal | 908801 | 60,448 | 8.09 | 422   | 15         | 4.3   | KRT2             | Intermediate filament, keratin |
| Keratin, type II cytoskeletal 8     | 181573    | 53,529 | 5.52 | 238   | 9          | 4.3   | KRT8, CYK8       | Cytoplasm, Intermediate filament, keratin, nucleus |
| Keratin, type II cytoskeletal 7     | 12803727  | 51,444 | 5.42 | 287   | 36         | 3.5   | KRT7             | Cytoplasm, intermediate filament, keratin |
| Transcription regulation            | 17402900  | 67,690 | 7.18 | 111   | 4          | 4.1   | FUBP1            | Nucleus |
| ETS translocation variant 5         | 221042722 | 65,643 | 5.69 | 174   | 9          | 2.6   | ERM              | Nucleus |
| Translation                         | 4506667   | 34,423 | 5.71 | 193   | 14         | 3.5   | RPLP0            | Cytoplasm, nucleus |
| Ribosomal protein P0                | 4507947   | 69,448 | 6.61 | 403   | 16         | 2.4   | YARS             | Cytoplasm |
| Heterogeneous nuclear ribonucleoprotein L | 11527777 | 64,617 | 8.49 | 173   | 6          | 3.9   | HNRNPL, HNRPL, P/OKcl.14 | Cytoplasm, nucleus |
| Heterogeneous nuclear ribonucleoproteins A2/B1 | 4504447 | 36,041 | 8.67 | 188   | 13         | 3.2   | HNRNPA2B1        | Cytoplasm, nucleus, spliceosome |
| Heterogeneous nuclear ribonucleoprotein K | 460789  | 51,325 | 5.13 | 88    | 12         | 2.0   | HNRNPK, HNRPK    | Cytoplasm, nucleus, spliceosome |
| Calcium ion binding protein         | 4502101   | 38,918 | 6.57 | 124   | 26         | 3.9   | ANXA1, ANX1, LPC1 | Cytoplasm, nucleus, cell membrane |
| Annexin A1                          | 56967118  | 36,634 | 8.32 | 210   | 13         | 2.1   | ANXA2            | Basement membrane, extracellular matrix |
| Signal transduction                 | 5803225   | 29,326 | 4.63 | 136   | 26         | 2.5   | YWHAE            | Cytoplasm |
| 14-3-3 protein epsilon              | 4507949   | 28,179 | 4.76 | 315   | 23         | 2.2   | YWHAB            | Cytoplasm |
| 14-3-3 protein β/α                  | 704416    | 49,851 | 7.70 | 229   | 19         | 3.9   | TUFM             | Mitochondron |
| Elongation factor                   | 4506221   | 53,270 | 7.53 | 127   | 6          | 3.3   | PSMD12           | Proteasome, nucleus, cytoplasm |
| Proteasome regulatory               | 285975    | 51,088 | 5.94 | 347   | 15         | 3.3   | RABGDIB          | Cytoplasm |
| Chromatin regulator                 | 20070220  | 73,322 | 5.88 | 43    | 1          | 3.2   | PRMT5            | Cytoplasm, nucleus |
| N-methyltransferase 5               | 48255891  | 60,110 | 4.34 | 51    | 8          | 2.6   | PRKCSH           | Endoplasmic reticulum |
| Glycan metabolism                   | 20070125  | 57,480 | 4.76 | 586   | 24         | 2.6   | P4HB             | Endoplasmic reticulum |
| Protein disulfide isomerase         | 62898301  | 42,857 | 5.90 | 167   | 9          | 2.3   | SERPIN           | Secreted |
L-plastin expression was identified in RMCCA1 cells cultured in matrix gel (Fig. 2B). A previous study demonstrated that L-plastin localizes to actin-rich membrane structures involved in locomotion, adhesion and immune defense, thereby implying that L-plastin is involved in the organization of the actin cytoskeleton (17). In addition, L-plastin has also been detected in solid tumors of epithelial and mesenchymal origin and has been suggested to be involved in cancer cell invasion (18). In line with these observations, we found that the number of cholangiocarcinoma cell invasion events significantly decreased when the expression of L-plastin was inhibited with L-plastin siRNA.

Table II. Correlation between L-plastin expression and the clinicopathological features of cholangiocarcinoma patients.

| Characteristics          | L-plastin expression | P-value |
|--------------------------|----------------------|---------|
|                          | Negative  | Positive |         |
| Gender                   | 8    | 5        | 1.00    |
| Male                     | 7        | 4        |         |
| Female                   | 10       | 6        |         |
| Tumor differentiation    | 5        | 3        | 1.00    |
| Well                     | 10       | 6        |         |
| Moderate and poor        | 4        | 3        | 0.65    |
| Lymph node metastasis    | 11       | 6        | 1.00    |
| No                       | 4        | 3        |         |
| Yes                      | 10       | 5        |         |
| Distant metastasis       | 5        | 3        | 1.00    |
| No                       | 10       | 5        |         |
| Yes                      | 11       | 6        |         |

Figure 3. Effect of L-plastin on cholangiocarcinoma cell invasion. (A) The expression levels of L-plastin and β-actin in RMCCA1 cells transfected with either control dsRNA or L-plastin siRNA were determined by western blotting. Lane 1 represents protein extracted from RMCCA1 cells treated with control dsRNA, and lane 2 represents protein extracted from RMCCA1 cells treated with L-plastin siRNA. (B) Immunofluorescence detection by confocal microscopy. The cells were transfected with either control dsRNA or L-plastin siRNA. The cells were then triple-stained with monoclonal L-plastin antibody (red), phalloidin (green, to reveal filamentous actin) or TOPO3 (blue, to reveal the nucleus). The scale bar indicates 10 µm. (C) Box plots comparing the number of cholangiocarcinoma cell invasion events in cholangiocarcinoma cells treated with the control (dsRNA) and L-plastin siRNA (P<0.001 by analysis of variance, compared with the control).

Effect of L-plastin on cholangiocarcinoma cell invasion. To determine whether the expression of L-plastin is associated with cholangiocarcinoma cell invasion, we knocked down the expression of L-plastin using L-plastin siRNA. The western blot (Fig. 3A) and immunofluorescence studies (Fig. 3B) demonstrated that L-plastin was significantly downregulated after transfecting the RMCCA1 cells with L-plastin siRNA. Moreover, the invasion assay showed that the number of cancer cell invasion events was significantly decreased with the L-plastin siRNA cells compared with those treated with the control dsRNA (P<0.001; Fig. 3C).

Detection of L-plastin expression in paraffin-embedded cholangiocarcinoma specimens. The expression of...
L-plastin localization in cholangiocarcinoma cells. We found that L-plastin was primarily expressed in mesenchymal-like cholangiocarcinoma cells. These findings suggest that L-plastin expression is associated with the epithelial-mesenchymal transition of cholangiocarcinoma cells.

To understand whether our in vitro findings are also relevant in vivo, we performed immunohistochemical analyses of tumor specimens derived from cholangiocarcinoma patients. Our analyses demonstrated that L-plastin is expressed in cholangiocarcinoma specimens. However, the level of expression was not significantly correlated with tumor differentiation, lymph node or metastatic status. This finding is in contrast to that reported for colorectal cancer, in which the expression of L-plastin is significantly correlated with cancer staging (22). Variations in the biological features of the tumors and the limited number of specimens in our study may account for these differences. In conclusion, attachment to the ECM promotes cholangiocarcinoma cell progression by inducing L-plastin expression. Understanding this mechanism may help to identify a novel molecular target for the development of an effective therapy for cholangiocarcinoma patients.

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