การศึกษาการเปลี่ยนแปลงโปรแกรมลุกลามของเซลล์มะเร็งทางเดินน้ำดีโดยการเปลี่ยนแปลงสภาพแวดล้อมของเซลล์

นางสาวสุทธิดารักษ์ ชัยจันทร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเวชศาสตร์ (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2554

EFFECT OF EXTRACELLULAR ENVIRONMENTAL FACTORS IN CHOLANGIOCARCINOMA CELLS INVASIVENESS

Miss Suthidarak Chaijan

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biomedical Sciences
(Interdisciplinary Program)
Graduate School
Chulalongkorn University
Academic Year 2011

Thesis Title	EFFECT OF EXTRACELLULAR ENVIRONMENTAL FACTORS IN CHOLANGIOCARCINOMA CELLS INVASIVENESS
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สุทธิดารักษ์ ชัยจันทร์ : การศึกษาการเปลี่ยนแปลงโปรแกรมลุกลามของเซลล์มะเร็งทางเดินน้ำดีโดยการเปลี่ยนแปลงสภาพแวดล้อมของเซลล์.
(EFFECT OF EXTRACELLULAR ENVIRONMENTAL FACTORS IN CHOLANGIOCARCINOMA CELLS INVASIVENESS)
อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ศ.นพ.ดร. อภิวัฒน์ มุทิรางกูร, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ.นพ.ดร. กวิญ ลีละวัฒน์, ดร. สิทธิรักษ์ รอยตระกูล, 52 หน้า.

โรคมะเร็งทางเดินน้ำดีเป็นโรคมะเร็งที่มีความรุนแรงสูง มักมีการลุกลามไปยังอวัยวะข้างเคียง หรืออวัยวะห่างไกล เอกตร้าเซลลูล่าแมตริก (extracellular matrix, ECM) มีการแสดงทางพยาธิสภาพพบได้มากรอบเซลล์มะเร็งทางเดินน้ำดีแต่มันก็ไม่ได้มีหน้าที่เป็นแค่ตัวขวางกั้นมะเร็งที่จะมีการลุกลามเพียงเท่านั้น ในการศึกษาครั้งนี้ เราสนใจที่จะศึกษาความเกี่ยวข้องของเอกตร้าเซลลูล่าแมตริกกับโปรมแกรมลุกลามของเซลล์มะเร็งทางเดินน้ำดี เซลล์ไลน์ที่ใช้ในการศึกษาคือ RMCCA1 ได้ถูกเลี้ยงในเพลตที่มีและไม่มีเอกตร้าเซลลูล่าแมตริกซึ่งเตรียมจากแมตริกเจล (matrigel) และได้ทดสอบผลของการลุกลามของเซลล์มะเร็งเทียบกัน ซึ่งก็พบว่าเซลล์มะเร็งที่ได้จากเพลตแมตริกเจล มีการลุกลามที่เพิ่มขึ้นอย่างมีนัยสำคัญ รวมทั้งได้ศึกษาโปรตีนที่มีการแสดงออกเปลี่ยนแปลงไปเมื่อเซลล์ถูกเลี้ยงในแมตริกเจล โดยเทคนิคอิเลคโตโฟริสิสแบบสองระนาบ (2D gel electrophoresis) พบว่าโปรตีนหลายตัวมีการแสดงออกที่เพิ่มขึ้น L-plastin เป็นโปรตีนในกลุ่มของ actin-binding protein และ pyruvate kinase M2 (PKM2) เป็นโปรตีนในกลุ่มของ glycolytic pathway ที่มีการเพิ่มขึ้นอย่างมีนัยสำคัญ ซึ่งอาจเกิดจากการกระตุ้นของแมตริกเจล การยับยั้งการทำงานของ L-plastin และ PKM2 โดยใช้ siRNA สามารถลดการลุกลามของเซลล์มะเร็งทางเดินน้ำดีได้ จากข้อมูลนี้บ่งชี้ว่าเอกตร้าเซลลูล่าแมตริกน่าจะส่งเสริมการลุกลามของเซลล์มะเร็งทางเดินน้ำดีโดยมีการเพิ่มของ L-plastin และ PKM2 โปรตีน การค้นพบนี้น่าจะเป็นประโยชน์ในส่วนของการเปิดเผยกลไกการลุกลามและเป็นประโยชน์กับการหาหนทางยับยั้งการลุกลามของมะเร็งทางเดินน้ำดีต่อไป

สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต .....
ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....
ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....
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5087881320 : MAJOR BIOMEDICAL SCIENCES

KEYWORDS : CHOLANGIOCARCINOMA / EXTRACELLULAR MATRIX / CANCER CELL INVASION / L-PLASTIN / LC-MS/MS

SUTHIDARAK CHAIJAN : EFFECT OF EXTRACELLULAR ENVIRONMENTAL FACTORS IN CHOLANGIOCARCINOMA CELLS INVASIVENESS. ADVISOR : PROF. APIWAT MUTIRANGURA, CO-ADVISOR : ASST. PROF. KAWIN LEELAWAT, SITTIRUK ROYTRAKUL, 52 pp.

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 display an enrichment of the ECM surrounding the tumor cells. In this study, we examined the role that the ECM plays in the regulation of the invasiveness of cholangiocarcinoma cells. The cholangiocarcinoma cell line RMCCA1 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 (2D) gel electrophoresis and liquid chromatography-tandem mass spectrometry (LC-MS/MS). 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. Many proteins were induced by culturing the RMCCA1 cells in the matrix gel. The expression of L-plastin, an actin-binding protein, and pyruvate kinase M2 (PKM2), responsible for energy production within the glycolytic pathway, were significantly upregulated. The knockdown of L-plastin and PKM2 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 and PKM2. These findings suggest the potential exploitation of this mechanism as a means of inhibiting the invasiveness of cholangiocarcinoma cells.

Field of Study : Biomedical Sciences Student's Signature .....

Academic Year : 2011 Advisor's Signature .....

Co-advisor's Signature .....

Co-advisor's Signature……..

ACKNOWLEDGEMENTS

This dissertation could not have been written without the support of Department of Surgery, Rajavithi Hospital by Rajavithi Hospital Grant, and the Thailand Graduate Institute of Science and Technology (TGIST), the National Science and Technology Development Agency, and the CU. Graduate School Thesis Grant, Chulalongkorn University.

I would like to state my esteem to my advisor, Professor Apiwat Mutirangura, who inspired, motivated and advised me to complete this dissertation.

I would like to express my appreciation to my co-advisor, Dr. Kawin Leelawat, who encouraged, suggested and supported me to complete this dissertation and Department of Surgery, Rajavithi Hospital for facilities to do research.

I would like to acknowledge my co-advisor, Dr. Sittiruk Roytrakul, who prompted, stimulated and counseled me to complete this dissertation and The National Center for Genetic Engineering and Biotechnology for facilities to do research.

I would like to thank Ms. Siriluck Narong and Ms. Siriporn Keeratichamroen at Department of Surgery, Rajavithi Hospital, and Ms. Wandee Udomchaiprasertgul at Chulabhorn Research Institute for companionship and support, and Ms. Atchara Paemanee at The National Center for Genetic Engineering and Biotechnology for the mass spectrometry assistance.

My special thanks to Dr. Wanida Laiwattanapaisal, Dr. Poramaet Laowanapiban, Mr. Jittikhun Siripatananukul and Ms. Songsaeng Wingwart for their assistance to my dissertation.

Finally, I would like to express my deepest gratitude to my mother and my brother for their love and understanding which brought me today's success.

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LIST OF ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
ECM	Extracellular matrix
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
PSC	Primary sclerosing cholangitis
FN	Fibronectin
LN	Laminin
GAGs	Glycosaminoglycans
CH	Calponin homology
IEF	Isoelectric focusing
ACN	Acetonitrile
DTT	Dithiothreitol
IAA	Iodoacetamide
PKM2, M2-PK	Pyruvate kinase M2
GAPDH	Glyseraldehyde-3-phosphate dehydrogenase
IgG	Immunoglobulin G
siRNA	Small interfering ribonucleic acid

CHAPTER I

INTRODUCTION

Background and Rationale

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 habitants [3]. In the United States, the most commonly recognized risk factor for cholangiocarcinoma is primary sclerosing cholangitis (PSC) [4]. However, in Southeast Asia and especially in Thailand, infection with hepatobiliary flukes (*Opisthorchis viverrini*) is the most common risk factor for cholangiocarcinoma [5]. Therapeutic options for cholangiocarcinoma patients have been limited, as this type of cancer responds poorly to both chemotherapy and radiation therapy. Surgery is thus the only potentially effective treatment for cholangiocarcinoma. However, typical five-year survival rates of 32% to 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, e.g., fibronectin (FN), laminin (LN), collagen and heparin sulfate proteoglycans, communicate with cancer cells and modulate a variety of cellular functions required for cancer cells to display invasive and metastatic properties [9-11]. Many lines of evidence 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.

Research questions

1. Does ECM promote cholangiocarcinoma invasion?
2. Which are proteins or mechanisms that potential in cholangiocarcinoma invasion?

Objectives

1. To study the effects of ECM involved in cholangiocarcinoma invasion
2. To study the pathways or molecules that potential in cholangiocarcinoma invasion

Hypotheses

1. ECM may involve in cholangiocarcinoma invasion by promoting some protein expressions
2. Protein expression profile of cholangiocarcinoma can be found from proteomics study
3. Pretein expression of candidates may correlate to cholangiocarcinoma invasion

Key words

cholangiocarcinoma; extracellular matrix; cancer cell invasion; L-plastin; LC-MS/MS

Expected result

This study will help us to identify cholangiocarcinoma proteins mediated by extracellular environmental factors which can promote cancer cell invasion.

Conception framework

Research Question I: Does ECM promote cholangiocarcinoma invasion?

To study the effects of ECM involved in cholangiocarcinoma invasion

- The human cholangiocarcinoma cell line RMCCA1 was grown in BD Matrigel matrix. That is three-dimensional (3D) environments cells culture, where collagen fibers contact both ventral (lower) and dorsal (upper) surfaces of the cells.
- Invasion assay was observed the correlation between different types of ECM and time of incubation in ECM microenvironment. Control was the cells that were cultured on uncoated plates.

Research Question II: Which are proteins or mechanisms that potential in cholangiocarcinoma invasion?

1. To Identify invasion related proteins of Cholangiocarcinoma by proteomic analysis
    - Two-dimensional (2D) gel electrophoresis was performed for the analysis of proteins extracted from cholangiocarcinoma cells cultured in uncoated and 24 hr matrix-gel-coated plates.
    - Protein identification by Liquid chromatography-tandem mass spectrometry (LC-MS/MS)
2. To select candidate peoteins
    - Proteins involved actin binding protein (L-plastin) and energy metabolism (PKM2) were selected for investigation.
3. To investigate effect of candidate proteins on cholangiocarcinoma cell invasion
    - L-plastin and PKM2 protein were knocked down by using transient small interfering RNA transfection.
    - The invasion assay was used to observe cancer cell invasion events with the siRNA cells compared with those treated with the control dsRNA.

CHAPTER II

REVIEW OF RELATED LITERATURE

Cholangiocarcinoma is a cancer arising from bile duct epithelium. Thailand, especially in the North-East is the endemic area of cholangiocarcinoma. *Opisthorchis viverrini* are the major risk factors for the development of cholangiocarcinoma [15]. This disease is one of the most causes of cancer related death in Thailand [3]. The causes of lethalness of this disease are not only its rapid growth but also the tendency to invade adjacent organs and metastasize [16-19]. Only Surgery is the curative treatment for these patients. Complete resection of the tumor with negative histologic margins offers the best possibility of long-term survival. However, only a small number of patients can be achieved this operation [6-8]. Most of the patients are presenting with advance stages of disease which beyond surgical treatment. Consequently, diagnosis of cholangiocarcinoma at early stages of disease is very important. Unlike other kinds of solid tumors, a pathological diagnosis of cholangiocarcinoma is very difficult because of the location and the desmoplastic characteristic of this disease [17, 20]. For that reason, the diagnosis of cholangiocarcinoma is usually based on radiological imaging and tumor markers [21]. Pathological specimens indicate that cholangiocarcinoma samples display the growth of fibrous or connective tissue surrounding the tumor cells and then they may have exchanged signals that regulate local extracellular matrix (ECM) proteins, stimulate invasion and migration as well as promote cell proliferation and survival. Cholangiocarcinoma may use ECM for being malignancy.

The ECM provides a chemical and mechanical structure, which is essential for development and for responses to pathophysiological signals [22]. The ECM is a complex structural substance surrounding and supporting cells that are found within mammalian tissues. The ECM is often referred to as the connective tissue. The ECM is composed of 3 major classes of biomolecules: First, Structural proteins (collagen and elastin); Second, Specialized proteins (e.g. fibrillin, fibronectin, and laminin); Third, Proteoglycans: these are composed of a protein core to which is attached long chains of

repeating disaccharide units termed of glycosaminoglycans (GAGs) forming extremely complex high molecular weight components of the ECM. Due to its diverse nature and composition, the ECM can serve many functions, such as providing support and anchorage for cells, segregating tissues from one another, and regulating intercellular communication. The ECM regulates a cell's dynamic behavior. Formation of the extracellular matrix is essential for processes like growth, wound healing and fibrosis. Knowledge of ECM structure and composition also helps in comprehending the complex dynamics of tumor invasion and metastasis in cancer biology.

Cancer cell invasion is the active process of translocation of cancer cells across ECM barrier. Invasion requires adhesion, proteolysis of ECM components and migration. Cell invasion into the 3D ECM is multistep biophysical process involved in inflammation, tissue repair and metastasis [23]. To migrate, the cell body must modify its shape to interact with the surrounding tissue structures. By this, the ECM provides the substrate, as well as a barrier towards the progressing cell body. The protein–protein interactions and signaling events that underlie shape change and regulate cell migration are integrated in the concepts of focal adhesion dynamics and actomyosin polymerization and contraction [24]. Initial propulsion and elongation of leading pseudopods are driven by actin polymerization and assembly to filaments. Growing cell protrusions then touch the adjacent ECM and initiate binding via adhesion molecules, most notably transmembrane receptors of the integrin family. Depending on the cell type and ECM substrate, focal contact assembly and migration can be regulated by different integrins. These include $\alpha 5\beta 1$ integrin, which binds fibronectin; $\alpha 6\beta 1$ or $\alpha 6\beta 4$, which bind laminin; $\alpha v\beta 3$, which binds fibronetin or vitronectin; and $\alpha 2\beta 1$, which binds fibrillar collagen. Other, non-integrin receptors, such as CD44, discoidin receptors, CD26, immunoglobulin superfamily receptors, and surface proteoglycans, also interact with ECM components and signal cell motility. However, the generating role of these factors is not clearly established. The engagement of integrins and other adhesion receptors leads to the recruitment of surface proteases to ECM contacts and focalized proteolysis. ECM degradation occurs while the advancing cell body gains volume towards the ECM

scaffold and is likely to provide the space required for cell expansion and migration. During the focal contacts development, actin filaments locally elongate and assemble, through the action of crosslinking proteins such as α-actinin, myosin II and others. By several mechanisms, which are incompletely understood, cell-substrate linkages then resolve preferentially in the back of the cell, whereas the leading edge remains attached to the ECM and further elongates [25].

Fully mature focal contacts have only been observed in cells that are firmly attached to 2D substrates. The focal contacts can be formed by highly adhesive cells that express high levels of integrins. Conversely, when cells are placed in 3D substrates, clustered integrins tend to couple to less-completely assembled focal interactions and a predominantly cortical actin cytoskeleton, whereas stress fiber formation is rare [26]. Low-adhesion cells, such as lymphocytes and lymphoma cells, express low levels of ECM-binding integrins. In these cells, migration is sustained by cortical actin networks, and no focal contacts or stress fibers form [27].

In vitro and *in vivo* studies in cell lines led to the original observations that individual tumor cells are motile [28]. Based on cell type, integrin engagement, cytoskeletal structure and protease production, single-cell migration can occur in different morphological variants as diversity of tumor invasion mechanisms. These variants include mesenchymal and amoeboid types, as well as cell chains (Figure 1). Cells that undergo mesenchymal migration have a fibroblast-like spindle shaped morphology that is dependent on integrin mediated adhesion dynamics and the presence of high traction forces on both cell poles. Many established tumor cell lines do not follow these mesenchymal characteristics, but use a less adhesive, amoeboid type of migration [25].

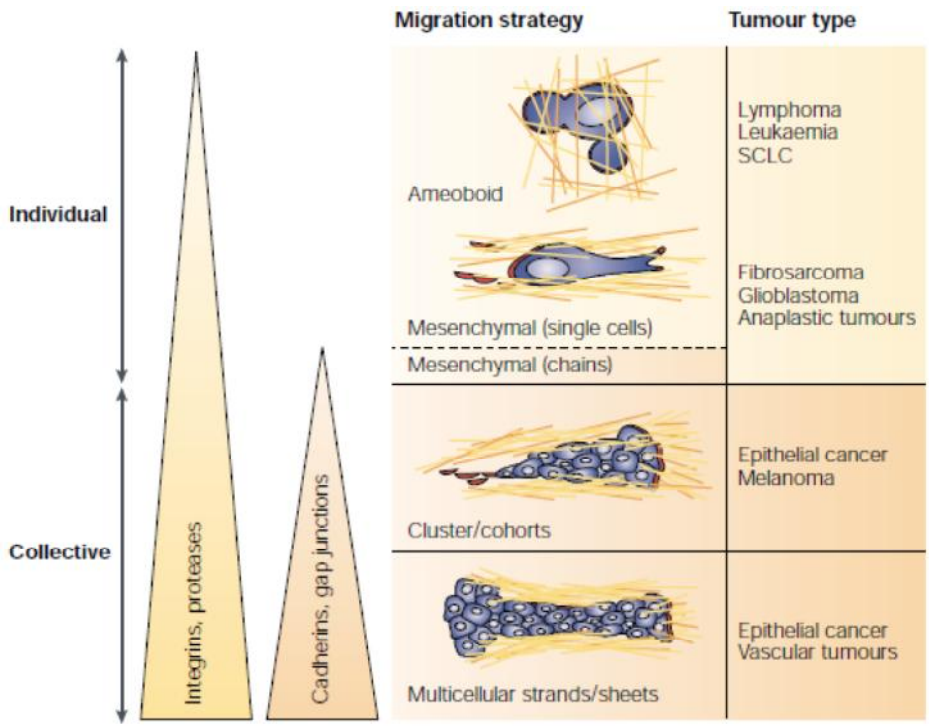


Figure 1. Diversity of tumor invasion mechanisms. Individual or collective tumor-cell migration strategies are determined by different molecular programs (triangles). From individual (top) to collective (bottom) movements, increased control of cell–ECM interaction is provided by integrins and matrix-degrading proteases. Cell–cell adhesion through cadherins and other adhesion receptors, as well as cell–cell communication, via gap junctions, are specific characteristics of collective cell behavior. Detached and disseminating cell collectives (clusters or cohorts) are observed in epithelial cancers that retain high or intermediate levels of differentiation, such as breast and colon carcinoma, prostate cancer, as well as melanoma. Multicellular strands and sheets that do not detach are invasive, yet rarely metastatic. These occur in some epithelial cancers, including basal-cell carcinomas and benign vascular tumors (Friedl P. and Wolf K., 2003) [25].

During the development of cancer, some cancer cells have the invasive ability to move out of the primary tumor site, invade to adjacent tissue, travel to the distant sites via the circulatory systems, and establish new colonies. This process called metastasis is the leading cause of death in cancer patients. Cancer cell invasion is certainly a prerequisite for metastasis. However, invasion and metastasis are excessively complex processes, and their genetic and biochemical determinants remain incompletely understood. Potentially, at the mechanistic level, operational strategies are involving changes in the physical coupling of cells to their microenvironment and activation of extracellular proteases. Typically, tumor cells invade the basement membrane by secreting enzymes that digest the ECM proteins. The ECM degradation proteases can be divided in to four different classes. Those are serine protease, cysteine protease, aspartate protease and metalloprotease. Among the list of enzymes involved in cancer, large information has been accumulated concerning the metalloprotease, known as matrix metalloproteinases (MMPs) [20]. A family of extracellular proteases called MMPs is essential for proper ECM remodeling. MMPs were considered to be important almost exclusively in invasion and metastasis [29]. French, J. J., et al. suggested that MMPs may play an important role in the degradation of extracellular matrix (ECM) and promotion of cholangiocarcinoma invasion [30].

Matrix metalloproteinases (MMPs) are zinc dependent endopeptidases, proteolytic enzymes. They play roles in the mechanisms of the turnover and degradation of ECM components and basement membranes. Historically, literatures demonstrated that degradation of ECM by MMPs is a key role in the mechanism of tumor invasion and metastasis [31]. MMPs are possibly involved in the processes of fetal development and normal tissue remodeling as well as wound healing and inflammation [32].

From previous studies, MMPs were considered to be important restrictively in invasion and metastasis. However, recent studies certify that MMPs are involved in several steps of cancer development. Their basic mechanisms of action are involved in protein degradation that regulates various cell behaviors with relevance to cancer biology. These include cancer cell growth, differentiation, apoptosis, migration and

invasion, and the regulation of tumor angiogenesis and immune surveillance. The MMPs are endopeptidases that can cleave virtually any component of extracellular matrix. MMP activity is tightly controlled by endogenous inhibitors. The best-studied endogenous MMP inhibitors are TIMPs-1, -2, -3 and -4 that reversibly inhibit MMPs in a 1:1 stoichiometric fashion [29].

The MMPs are classified according to their structure. There are eight distinct structural classes of MMPs: five are secreted and three are membrane-type MMPs (MT-MMPs). The MMPs are synthesized as inactive enzyme precursors (pro-MMPs). They are kept inactive by an interaction between a cysteine-sulphydryl group in the propeptide domain and zinc ion bound to the catalytic domain. The enzyme activation requires proteolytic removal of the propeptide prodomain [29].

Evidently, cancer invasion may involve in actin-bundling proteins that is proteins capable of cross-linking actin filaments into networks or bundles play an important role in the assembly of cellular actin cytoskeleton. L-plastin is a member of a large family of actin-cross-linking protein, including **α**-actinin and filamin [33]. Then, those proteins are expected to discover in this study.

Plastins belong to a class of actin-bundling proteins, and they are conserved from lower eukaryotes to humans. Three isoforms have been characterized in mammals: T-plastin is expressed in cells from solid tissue, whereas L-plastin occurs predominantly in hematopoietic cells. The third isoform, I-plastin, is specifically expressed in the small intestine, colon and kidney (Figure 2). These proteins share the unique property of cross-linking actin filaments into tight bundles [34]. Interestingly, the hematopoietic isoform of the plastins, L-plastin, is not only expressed by hematopoietic cells, but also by most human cancer cell lines. L-plastin is considered to be a valuable marker for cancer. L-plastin has a common domain structure consisting of two amino-terminal EF-hands, implicated in calcium-binding, followed by two tandem actin-binding domains, each composed out of two calponin homology (CH) domains (Figure 3) [35]. In prostate carcinoma studies, expression of an L-plastin antisense gene in prostate carcinoma cell lines reduced the growth rates as well as invasion and motility. These data indicated that

L-plastin overexpression might be involved in prostate cancer invasion [36]. Evidently, the amount of L-plastin expression correlates with tumor stages in human colorectal cancer. It was concluded that the amount of L-plastin expression may represent a potential biomarker in human colorectal progression and metastasis [37]. Some evidences support that phosphorylation of L-plastin may build up specific actin structures in cells. Phosphorylation of L-plastin switches the protein from a low-activity to a high-activity state. Then, it might act as a coordinated manner of signals controlling the assembly of actin cytoskeleton and cell motility in a 3D-space [33]. From evidence, phosphorylation of L-plastin may influence the adhesiveness, the motility and invasiveness of human melanoma cells, key features of metastasis [35].

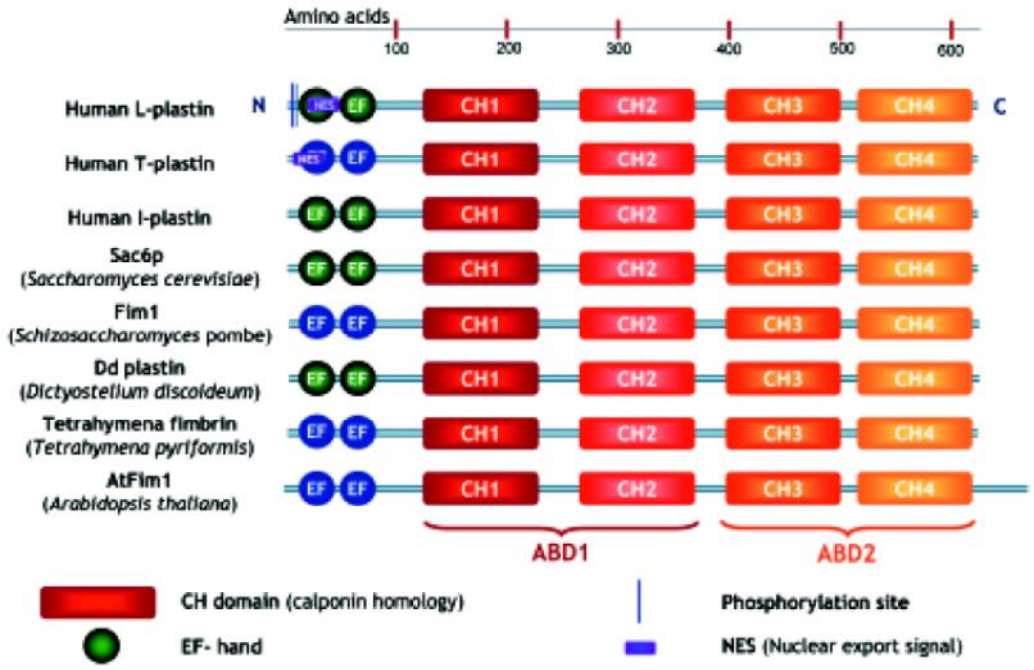


Figure 2. Domain organization in plastin isoforms from different species. Amino acids scale is drawn on top of the figure. Domains in blue have no or less functionality. ABD is actin-binding domain (Delanote V. *et al*., 2005) [34].

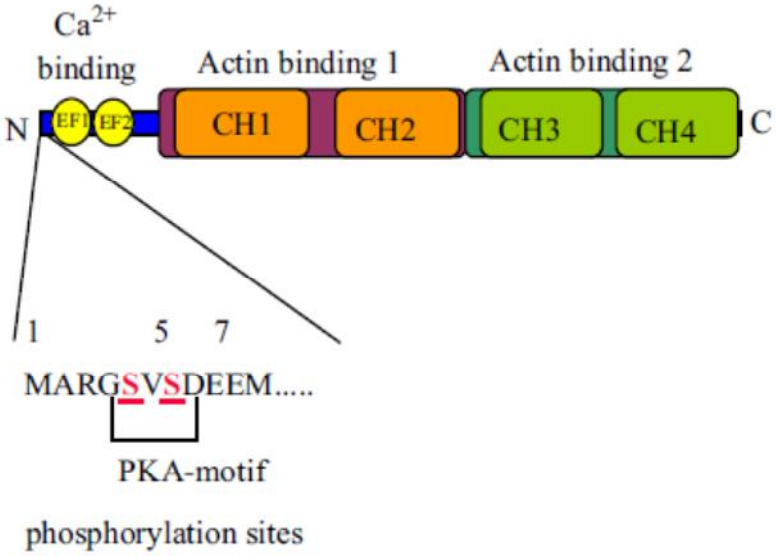


Figure 3. Functional domains in the L-plastin molecule. L-plastin have 2 N-terminally located phosphorylation sites (Ser 5 and Ser 7), 2 calcium-binding domains of the EF-hand type and 2 tandem organized actin-binding domains (Samstag Y. and Klemke M., 2007) [35].

According to cancer cell may require energy across ECM barrier, then ATP synthase may up-regulated to provide cell proliferation and invasion process. From the evidence, independent of whether mitochondrial respiration is low or not, cancer cells do exhibit high rate of glycolysis, was first noted by Otto Warburg more than 75 year ago (Figure 4) [38, 39]. Thus, proteins in glycolysis pathway are expected to increase as well, such as phosphoglycerate kinase, pyruvate kinase, lactate dehydrogenase. A key glycolytic enzyme that is consistently altered during tumorigenesis is pyruvate kinase isoenzyme M2 (PKM2, M2-PK). The M2 splice isoform of the glycolytic enzyme pyruvate kinase is necessary for the shift in cellular metabolism to aerobic glycolysis and that this promotes tumorigenesis [40]. In tumor cells, the dimeric form of PKM2 is always predominant. The dimerization is caused by direct interaction of PKM2 with certain oncoproteins. The oscillation between the tetrameric and dimeric form of PKM2 is absolutely necessary for tumor cells survival in environments with varying oxygen and nutrient supply. The lung PKM2 always consists of four equal subunits, whereas the PKM2 isoenzyme found in tumor cells is usually dimeric. A large glycolytic capacity together with the presence of PKM2 allows tumor cells to survive under hypoxic conditions and to migrate [41]. Phosphotyrosinebased regulation of PKM2 enzymatic activity may provide a direct link between cell growth signals using tyrosine kinases and control of glycolytic metabolism. Recent studies demonstrate that a cancer-cell-specific isoform of the enzyme PKM2 is regulated by binding to phospho-tyrosine motifs and further increased cell growth and tumor development. PKM2 enhances the use of glycolytic intermediates for macromolecular biosynthesis and tumor growth [42-44].

Although ECM has been known to play an important role in cancer cell invasion and migration, until now, it has not yet been studied of ECM mediated pathways in cholangiocarcinoma model.

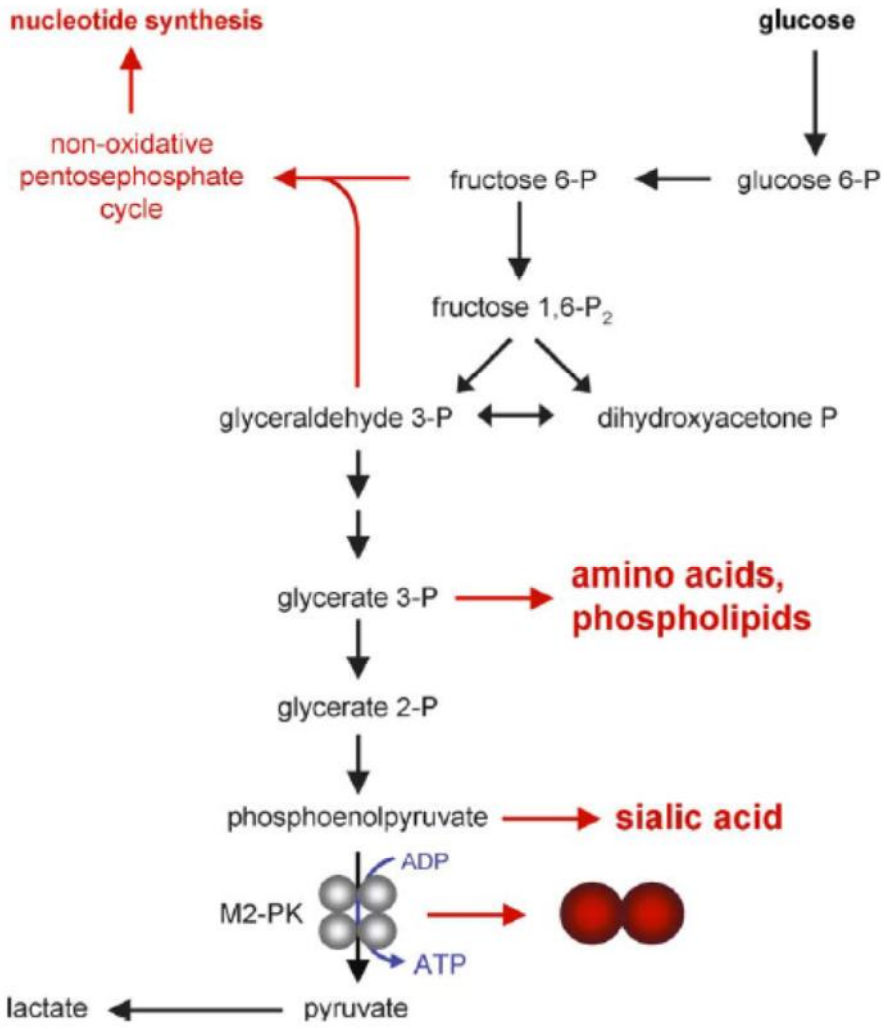


Figure 4. Glycolysis and its debranching synthetic pathways. Pyruvate kinase is responsible for net ATP production (Mazurek S. *et al.*, 2005) [41].

CHAPTER III

MATERIALS AND METHODS

Cell cultures

The human cholangiocarcinoma cell line RMCCA1, originally derived from a cholangiocarcinoma patient [45], 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% CO_2 humidified atmosphere.

Cell invasion assay

To study the mechanism of cancer cell invasion *in vitro*, RMCCA1 cells were cultured in Non-coated plates, Collagen Type I (0.05 mg/ml in 0.2 mg/ml gelatin), Collagen Type 4 (0.05 mg/ml in 0.2 mg/ml gelatin), Fibronectin (0.05 mg/ml in 0.2 mg/ml gelatin) and BD Matrigel matrix (0.2 mg/ml) (BD Biosciences, Bedford, MA, USA) for 24 hr. 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 hr was assessed by staining the cells with crystal violet [46]. The RMCCA1 cells were cultured in Matrix gel-coated plates for 0-24 hr to study pre-incubation time in matrix gel before invasion assay. Controls were the cells that were cultured on Non-coated plates for each time point of collection. Paired-Samples t-test was used for statistical analysis, and P values <0.05 were considered to be significant.

Gelatin-zymography

To detect Matrix metalloproteinase (MMP)-9 and MMP-2 activity, cell culture medium (serum free) was collected and concentrated. Electrophoresis was performed using zymogram gelatin gels. Samples, supernatant media from cholangiocarcinoma cells cultured in uncoated and matrix-gel-coated plates for 6 hr and 24 hr, mixed with sample buffer (Bio-Rad, Hercules, CA, USA) and stood at room temperature for 10 min without mercaptoethanol added and heated. Gelatin zymography was performed on a 7.5%

sodium dodecyl sulfate-acrylamide gel containing 0.1% gelatin, electrophoresis run at constant voltage 150 V for 1 hr, rinsed in dd-H_2O followed by incubation with bulky volume of renaturation buffer (2.5% TX-100 in 50 mM Tris-HCL pH 7.5) at room temperature for 1 hr with gentle shaking. The enzyme activity was developed in 50 mM Tris-HCL pH 7.5, 0.15 M NaCl, 10 mM $CaCl_2$ at 37°C for 18 hours and stained with Coomassie Blue (0.5% (w/v) Coomassie R250 in Methanol : Acetic acid : ddH_2O (50 : 10 : 40)). Then, it was destained with 7% Methanol and 5% Acetic acid. MMP-9 and MMP-2 activity images were obtained with ImageScanner (GE Healthcare, Uppsala, Sweden). Areas of enzymatic activity appeared as clear bands over the dark background.

Protein samples preparation for proteomics assay

Four hundred thousand cells were seeded in 60 mm cell dishes and cultured for 24 hours in Non-coated and Matrix gel-coated plates. After incubation, cells were washed twice with 25 mM sucrose and scraped in the lysis buffer containing 7 M Urea, 2 mM Thiourea, 4% CHAPS, 60 mM Dithiothreitol (DTT) and protease inhibitor cocktail. The cell suspension was transferred to microtube and sonicated at 4°C for 2 cycles, 30 sec each. Cells lystes were centrifuged at 12,000 rpm at 4°C for 10 min. Supernatant was collected for protein preparation using 2-D Clean-Up kit (GE Healthcare) following the manufacturer's protocol. Protein concentration was determined by Bio-Rad Bradford protein assay (Bio-Rad) using BSA as protein standard.

2D gel electrophoresis

Two-dimensional (2D) gel electrophoresis was performed for the analysis of proteins extracted from cholangiocarcinoma cells cultured in uncoated and 24 hr 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 IPG gel strips, pH 3-10 L (GE Healthcare) 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 using the Ettan DALT*six* Electrophoresis System. 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.

2D Image analysis

The proteins were visualized using an ImageScanner (GE Healthcare). The gel images were analyzed to determine differential protein expression profiles using the ImageMaster 2D Platinum software (GE Healthcare). Student's t-test was used for statistical analysis, and *P* values <0.05 were considered to be significant.

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 (GI), BIOTEC, National Science and Technology Development Agency, Pathumthani, Thailand. After 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 hr and alkylated at room temperature for 1 hr in the dark in the presence of 100 mM iodoacetamide (IAA) in 10 mM ammonium bicarbonate. After alkylation, the gel pieces were dehydrated twice with 100% ACN for 5 min. For the in-gel digestion of the proteins, 10 µl of a 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 of 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 of 50% ACN in 0.1% formic acid (FA) 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 and stored at -80°C until further mass spectrometric analysis.

LC-MS analysis

The LC-MS/MS analysis of the digested peptide mixtures was performed using a Waters SYNAPT™ HDMS™ system. The 1D-nanoLC was performed with a Waters nanoACQUITY UPLC system. Tryptic digests (4 μl) were injected onto an RP analytical column (20 cm × 75 μm) packed with a 1.7-μm Bridged Ethyl Hybrid (BEH) C18 material (Waters). The peptides were eluted with a linear gradient of 2% to 40% acetonitrile developed over 30 minutes 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) 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 Ions web-based search functionality at the National Center for Biotechnology Information non-redundant (NCBInr) database (www.matrixscience.com). The default search parameters were applied as follows: enzyme = trypsin; taxonomy = *Homo sapiens* (human); max. missed cleavages = 1; fixed modifications = carbamidomethyl (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. In Mascot, the ions score for an MS/MS match was based on the calculated probability, P, that the observed match between the experimental data and the database sequence was a random event. The reported score was -10Log(P). Individual ions scores from calculation indicated identity or extensive homology ($p<0.05$).

Western blot analysis

Protein extracts isolated from the cells cultured in the uncoated and 24-hr matrix gel-coated plates were separated by 12% SDS-PAGE and then transferred onto a nitrocellulose membrane. The membrane was subsequently incubated with monoclonal

antibodies against L-plastin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), pyruvate kinase M2 (PKM2), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin (Cell Signaling Technology, Danvers, MA, USA). Horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) and anti-rabbit IgG were used as secondary antibodies (GE Healthcare). The blots were visualized using an ECL Plus detection kit and Hyperfilm ECL (GE Healthcare).

Inhibition of L-plastin and PKM2 expression using transient small interfering RNA transfection

L-plastin and PKM2 siRNA (Santa Cruz Biotechnology) was used to knock down L-plastin and PKM2 gene expression. A fluorescein-labeled, double-stranded RNA duplex (BLOCK-iT™ Fluorescent Oligo, Invitrogen, NY, USA) was designed as a control. The siRNA molecules were diluted in Opti-MEM® I Medium without serum (Gibco) and mixed gently. Next, Lipofectamine™ 2000 (Invitrogen) was diluted in Opti-MEM® I Medium without serum, mixed gently and incubated for 5 minutes at room temperature. The diluted siRNA molecules and diluted Lipofectamine™ 2000 were then combined. The mixtures were incubated for 20 minutes at room temperature to allow for complex formation to occur. The siRNA molecule-Lipofectamine™ 2000 complexes were added to each well containing cells and medium and mixed gently by rocking the plate back and forth. The cells were incubated at 37°C in a CO_2 incubator for 6 hr. Next, the growth medium was replaced after 6 hr, and the cells were harvested after 24 hr following transfection. Western blotting analysis using the L-plastin antibody was performed to assess the degree of L-plastin gene expression knockdown.

Immunofluorescence microscopy

Cells (5×10^4) were washed 2 times in PBS, fixed with 4% Paraformaldehyde for 30 min and blocked with 4% BSA for 30 min. Then cells were incubated with the primary antibody, anti-L-Plastin, for 1 hr at room temperature. The cells were then washed and incubated with the appropriate secondary antibody (Alexa Fluor 594, anti-mouse, Molecular Probes, NY, USA) for 1 hr at room temperature. The actin filaments (F-actin) in the cell cytoplasm were stained with Alexa Fluor 488 phalloidin (Molecular Probes), and

the nuclei were stained with TOPO3. The cover slides were removed from the plates and mounted with antifade on the slides. Cell images were taken with a confocal scanning biological microscope (FV1000, Olympus, Tokyo, Japan).

Statistical analysis

Continuous values for the observed levels in the invasion assay were expressed as the mean and SD. The one-way ANOVA test was used for the analysis of the multiple variables of the cell invasion assay. Paired-Samples t-test enabled to determine whether the means of paired samples are equal. Student's t-test was employed to evaluate the mean differences in the intensity volume of each corresponding spot between the two groups of samples. The *P* value <0.05 was considered significant.

CHAPTER IV

RESULTS

Culturing cholangiocarcinoma cells in matrix gel increased their invasiveness

RMCCA1 cholangiocarcinoma cells were pre-incubated 24 hr in Collagen I, Collagen IV, Fibronectin and matrix gel before invasion assay were investigated. The result demonstrated that the number of cholangiocarcinoma cells that were cultured in matrix gel significantly invaded through the insertion cup than others (Figure 5). Then, RMCCA1 cells were incubated in matrix gel for 0-24 hr, 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 than observed with the cells that were cultured on uncoated plates (P<0.001) (Figure 6A and 6B).

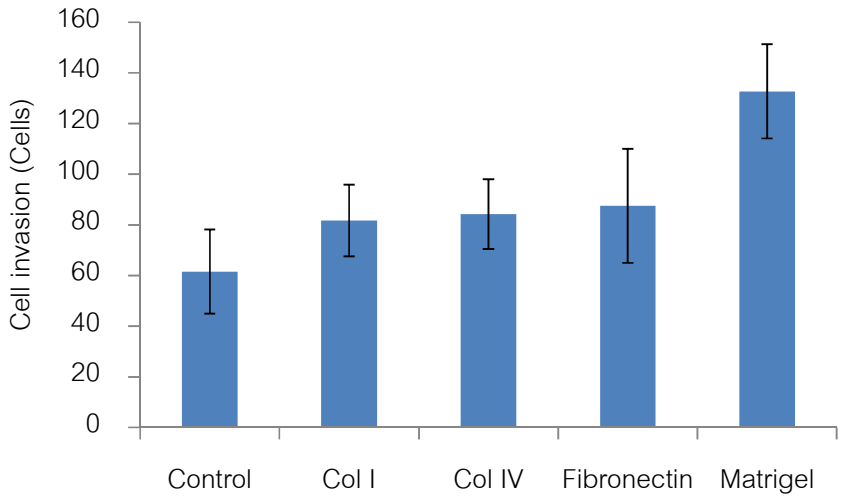


Figure 5. RMCCA1 cholangiocarcinoma cell invasion assays. The invasion assay comparing between cancer cells that pre-incubation in Collagen I, Collagen IV, Fibronectin and matrix gel before invasion assay were investigated. Control was the cells that were cultured on uncoated plates.

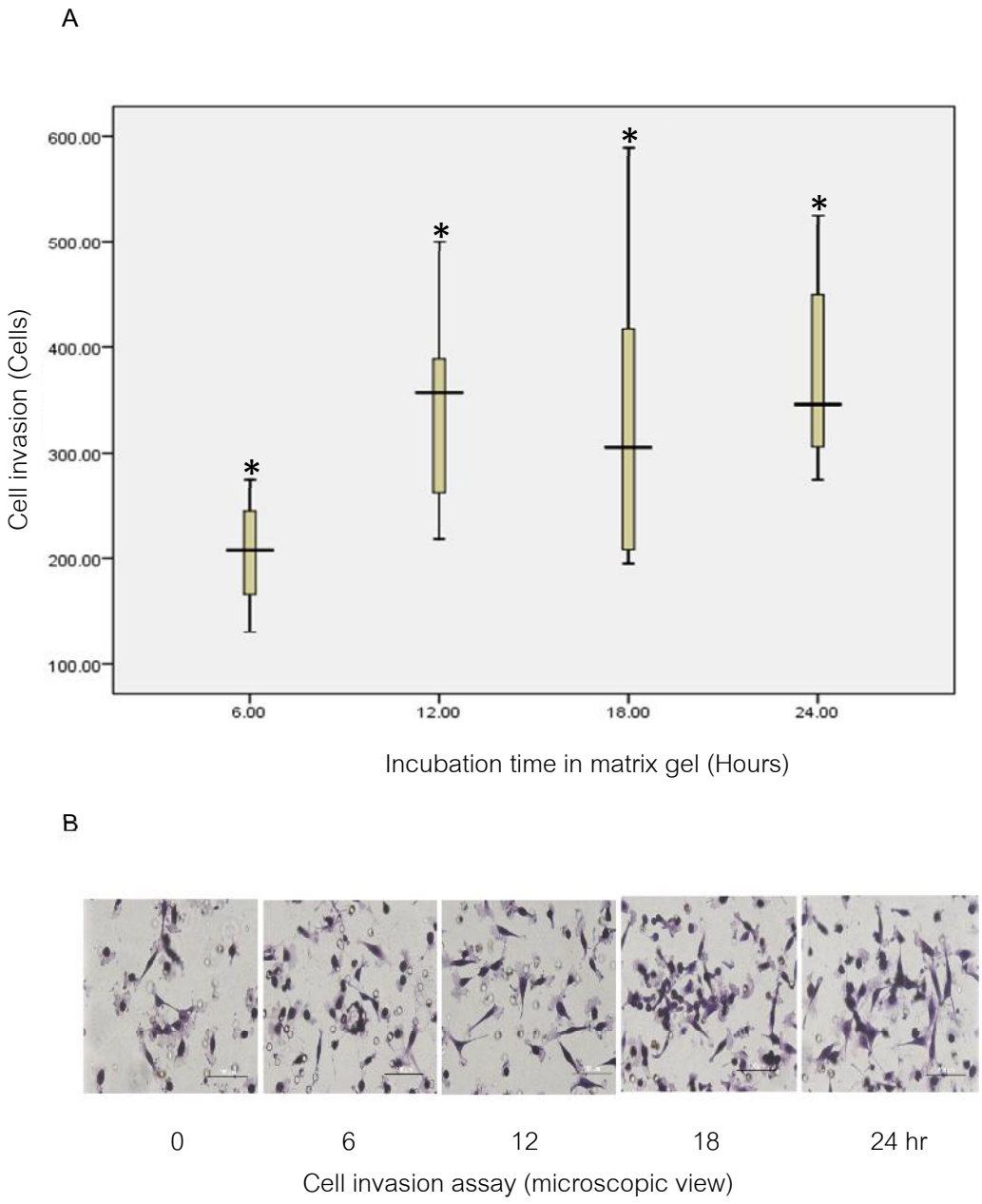


Figure 6. RMCCA1 cholangiocarcinoma with pre-incubated in matrix gel and cell invasion assays. **A)** Box plots comparing the numbers of cholangiocarcinoma cell invasion events in cells cultured in matrix gel and controls (*; Paired-Samples t-test; $P<0.001$ compared with each control). **B)** Micrographs of cholangiocarcinoma cell invasion (cholangiocarcinoma cells were cultured in matrix gel for 0, 6, 12, 18, or 24 hr before the invasion assays were performed). The scale bar indicates 100 μm.

MMP expression

To determine Matrix metalloproteinase (MMP)-9 and MMP-2, we found MMP-9 band at 92 kDa and MMP-2 at 72 kDa. For MMP-2 expressions among non-coated and matrigel coated plates were not much different. For MMP-9, incubation time at 24 hr in both non-coated and matrigel coated plates were higher than 6 hr incubation. And MMP-9 expressions in non-coated plates were higher than matrigel coated. Controls were 10% serum, non-coated and matrigel coated plates in 1% serum (Figure 7 and 8).

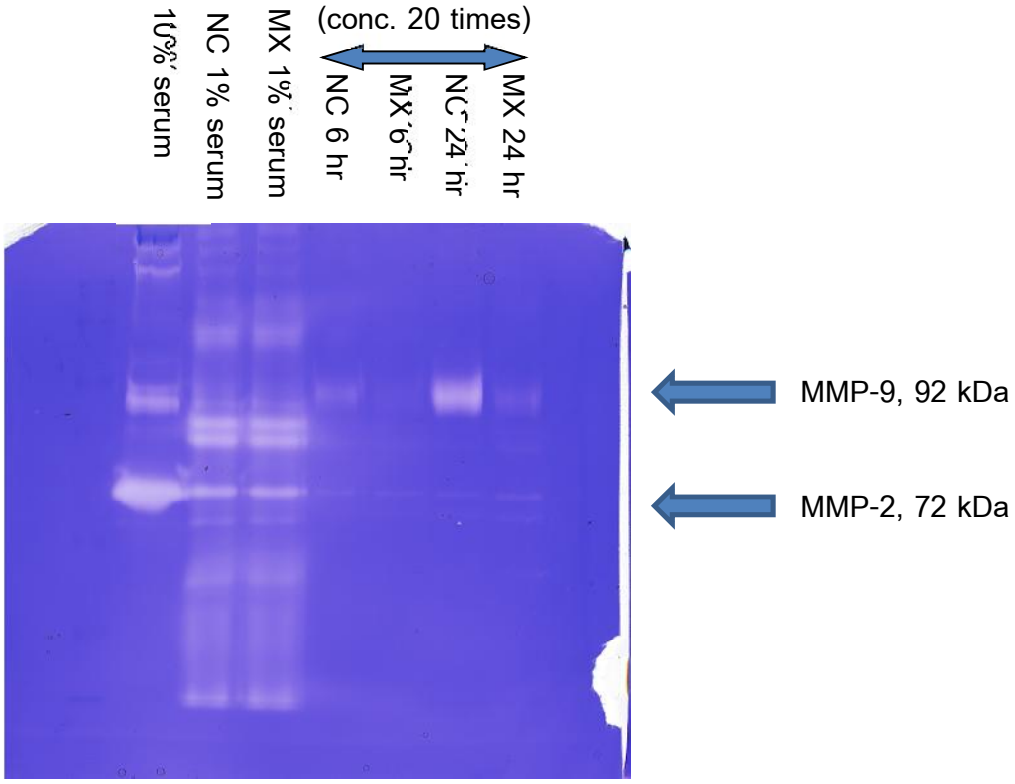


Figure 7. Gelatin zymography gel. MMP-9 and MMP-2 was found at 92 kDa and 72 kDa respectively. MMP-9 expressions in non-coated plates were higher than matrigel coated. However MMP-2 expressions were similar among the samples.

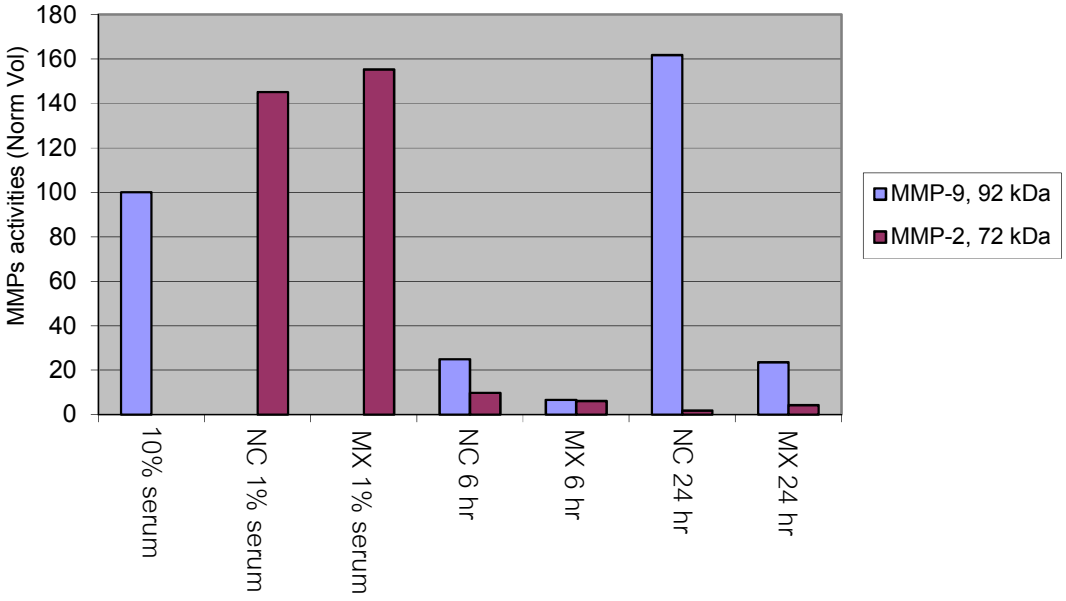


Figure 8. Bar plot from Gelatin zymography gel for MMPs. Non-coated plates at incubation time 24 hr were shown highest MMP-9 expression compare to other samples. Controls were 10% serum, non-coated and matrigel coated plates in 1% serum.

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, two-dimensional (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 (Figure 9). 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, 72 proteins displayed greater than twofold upregulation as determined by mass spectrometry. All the identified proteins were in the expected ranges of their theoretical molecular masses and pI values (Table 1).

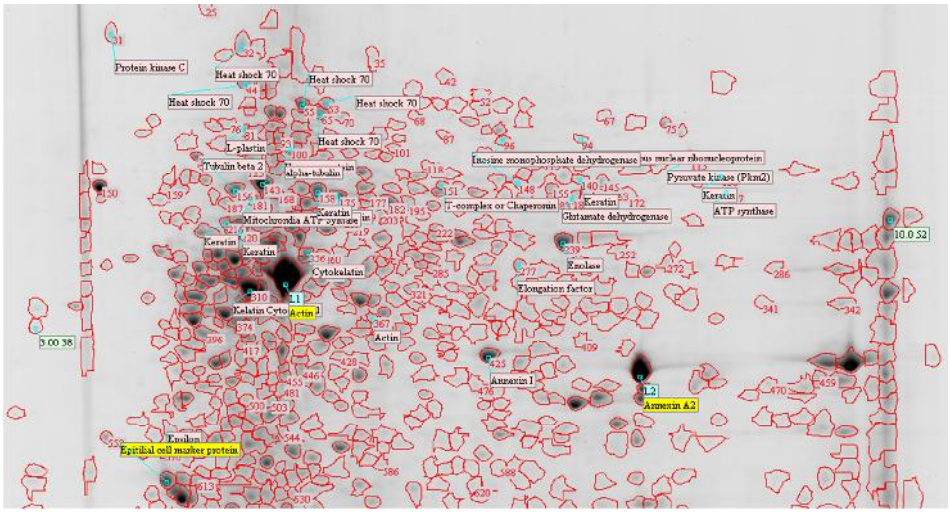


Figure 9. Two-dimensional (2D) gel electrophoresis and proteins of interest have been identified by mass spectrometry.

Table 1. A summary of upregulated proteins expressed in cholangiocarcinoma cells cultured in matrix gel, as identified by Q-TOF MS and MS/MS analyses.

NO.	GI number	Functional category and Protein Name	Mr	pI	Score	%Coverage	Ratio	GeneID	Cellular component
		Actin-binding protein							
1	62087548	L-plastin	56,196	5.21	52	6	4.6	LCP1, PLS2	Cytoplasm, Cell membrane, Cytoskeleton
2	340217	Cytovillin 2 (Ezrin)	68,233	5.80	335	13	3.9	VIL2, EZR	Cytoplasm, Cell membrane, Cytoskeleton
3	5031573	ARP3 actin-related protein 3 homolog	47,797	5.61	150	6	3.5	ACTR3	Cytoskeleton
4	2804273	Alpha-actinin-4	102,661	5.27	72	2	3.0	ACTN4	Cytoplasm, Nucleus
5	116241280	Adenylyl cyclase-associated protein 1	52,222	8.27	54	10	2.6	CAP1	Cell membrane
6	4507115	Fascin	55,123	6.84	160	19	2.5	FSCN1	Cytoplasm, Cytoskeleton
7	5031635	Cofilin-1	18,719	8.22	129	17	2.3	CFL1	Cytoplasm, Nucleus, Cell membrane, Cytoskeleton
		Energy metabolism							
8	67464392	Pyruvate Kinase (Pkm2)	60,277	8.22	166	20	7.3	PKM2	Cytoplasm, Nucleus
9	35505	pyruvate kinase	58,411	7.58	48	2	4.4	pyruv_kin	Cytoplasm, Nucleus
10	189998	M2-type pyruvate kinase	58,447	7.95	102	4	3.8	pyruv_kin	Cytoplasm, Nucleus
11	119598291	pyruvate kinase, muscle, isoform CRA_b	37,876	5.97	110	6	2.5	PKM2	Cytoplasm, Nucleus
12	4505763	Phosphoglycerate kinase 1	44,985	8.30	439	20	6.3	PGK1, PGKA	Cytoplasm
13	4505763	phosphoglycerate kinase 1	44,985	8.30	150	15	5.0	PGK1	Cytoplasm
14	28614	Aldolase A	39,706	8.34	267	11	3.5	ALDOA, ALDA	Cytoplasm, Nucleus
15	693933	Alpha-enolase (Phosphopyruvate hydratase)	47,421	7.01	148	22	2.4	ENO1	Cytoplasm, Nucleus, Cell membrane
16	126047	L-lactate dehydrogenase A chain	36,950	8.44	85	18	2.3	LDHA, PIG19	Cytoplasm
17	4757810	ATP synthase, H+ transporting, mitochondrial F1 complex	59,828	9.16	182	17	13.5	ATP5A1, ATP5F1	Mitochondrion, Mitochondrion inner membrane
18	32189394	mitochondrial ATP synthase beta subunit precursor	56,525	5.26	181	13	4.4	ATP5B	Mitochondrion, Mitochondrion inner membrane
19	643589	Dihydrolipoamide succinyltransferase	48,896	8.90	165	7	3.8	DLST	Mitochondrion
20	33337556	Citrate synthase	51,942	8.45	169	5	3.4	CS	Mitochondrion
21	182794	Fumarate hydratase, mitochondrial	50,524	7.23	305	11	2.7	FH	Mitochondrion, Cytoplasm
22	4885281	Glutamate dehydrogenase	61,701	7.66	197	16	3.9	GLUD1	Mitochondrion
23	83753870	Dihydrolipoamide dehydrogenase	50,656	6.50	236	9	2.2	DLD	Mitochondrion
24	76496475	acyl-Coenzyme A dehydrogenase	68,414	8.76	233	10	2.1	ACADVL	Mitochondrion, Mitochondrion inner membrane
25	37267	Transketolase	68,435	7.90	102	16	2.8	TKT	Cytoplasm, Nucleus

Table 1. Continued

NO.	GI number	Functional category and Protein Name	Mr	pI	Score	%Coverage	Ratio	GeneID	Cellular component
		Molecular chaperone							
26	74755280	Tumor rejection antigen 1, Endoplasmin	92,282	4.77	89	4	11.9	GRP94, TRA1, HSP90B1	Endoplasmic reticulum
27	62088648	tumor rejection antigen (gp96) 1 variant	66,140	5.08	261	9	4.2	HATPase_c	Endoplasmic reticulum
28	14124984	T-complex protein 1 subunit gamma	60,934	6.10	157	7	6.3	CCT3, CCTG	Cytoplasm
29	4502643	chaperonin containing TCP1, subunit 6A isoform a	58,444	6.23	412	15	3.4	CCT6A	Cytoplasm
30	5453603	chaperonin containing TCP1, subunit 2	57,794	6.01	170	8	3.4	CCT2	Cytoplasm
31	154146191	Heat shock protein HSP 90-alpha	85,006	4.94	163	5	5.2	HSP90AA1	Cytoplasm
32	119602173	Heat shock protein HSP 90-beta	57,868	4.92	59	2	2.3	HSP90AB1	Cytoplasm
33	5803181	Stress-induced-phosphoprotein 1 (Hsp70/Hsp90-organizing	63,227	6.40	145	19	3.8	STIP1	Cytoplasm, Nucleus
34	77702086	60 kDa heat shock protein	61,346	5.70	580	16	5.0	HSPD1, HSP60	Mitochondrion
35	386785	Heat shock protein	70,110	5.42	404	10	4.4	HSPA1L	Cytoplasm
36	5729877	Heat shock 70 kDa protein 8	71,082	5.37	203	13	3.8	HSPA8	Cytoplasm
37	21264428	Stress-70 protein, mitochondrial	73,920	5.87	70	6	3.7	HSPA9	Mitochondrion
38	386758	78 kDa glucose-regulated protein	72,185	5.03	251	7	4.1	GRP78, HSPA5	Endoplasmic reticulum
39	36796	T-complex polypeptide 1	60,869	6.03	197	9	4.0	TCP1	Cytoplasm, Cytoskeleton
40	15214852	Nucleophosmin (nucleolar phosphoprotein B23, numatrin)	32,760	4.64	198	12	3.3	NPM1, NPM	Cytoplasm, Nucleus, Cytoskeleton
41	4757900	Calreticulin	48,283	4.29	109	12	2.3	CALR	Cytoplasm, Endoplasmic reticulum, Extracellular matrix, Secreted
		Structural molecule							
42	5174735	Tubulin, beta, 2	50,255	4.79	282	14	3.8	TUBB2C, TUBB4B	Cytoplasm, Cytoskeleton, Microtubule
43	37492	Alpha-tubulin	50,810	5.02	123	13	2.6	TUBA4A, TUBA1	Cytoplasm, Cytoskeleton, Microtubule
		Cytoskeleton function							
44	4557701	Keratin, type I cytoskeletal 17	48,361	4.97	303	28	3.7	KRT17	Cytoplasm, Intermediate filament, Keratin
45	47939651	KRT17 protein	40,520	4.90	164	12	2.0	KRT17	Cytoplasm, Intermediate filament, Keratin
46	30311	Keratin, type I cytoskeletal 18	47,305	5.27	558	28	3.5	KRT18, CYK18, PIG46	Cytoplasm, Intermediate filament, Keratin
47	24234699	Keratin, type I cytoskeletal 19	44,079	5.04	455	44	2.1	KRT19	Intermediate filament, Keratin
48	5031839	Keratin, type II cytoskeletal 6A	60,293	8.09	248	29	5.8	KRT6A	Intermediate filament, Keratin

Table 1. Continued

NO.	GI number	Functional category and Protein Name	Mr	pI	Score	%Coverage	Ratio	GeneID	Cellular component
49	46812692	Keratin 6A	60,323	7.59	306	23	2.0	KRT6A	Intermediate filament, Keratin
50	908801	Keratin, type II cytoskeletal 2 epidermal	60,448	8.09	422	15	4.3	KRT2	Intermediate filament, Keratin
51	181573	Keratin, type II cytoskeletal 8	53,529	5.52	238	9	4.3	KRT8, CYK8	Cytoplasm, Intermediate filament, Keratin, Nucleus
52	181400	cytokeratin 8	53,656	5.30	291	32	3.0	cytokeratin 8	Cytoplasm, Intermediate filament, Keratin, Nucleus
53	12803727	Keratin, type II cytoskeletal 7	51,444	5.42	287	36	3.5	KRT7	Cytoplasm, Intermediate filament, Keratin
54	67782365	keratin 7	51,411	5.40	203	24	2.3	KRT7	Cytoplasm, Intermediate filament, Keratin
		Transcription regulation							
55	17402900	Far upstream element-binding protein 1	67,690	7.18	111	4	4.1	FUBP1	Nucleus
56	221042722	ETS translocation variant 5	65,643	5.69	174	9	2.6	ERM	Nucleus
		Translation							
57	4506667	Ribosomal protein P0	34,423	5.71	193	14	3.5	RPLP0	Cytoplasm, Nucleus
58	4507947	Tyrosyl-tRNA synthetase	59,448	6.61	403	16	2.4	YARS	Cytoplasm
59	11527777	Heterogeneous nuclear ribonucleoprotein L	64,617	8.49	173	6	3.9	HNRNPL, HNRPL, P/CKcl.14	Cytoplasm, Nucleus
60	4504447	Heterogeneous nuclear ribonucleoproteins A2/B1	36,041	8.67	188	13	3.2	HNRNPA2B1	Cytoplasm, Nucleus, Spliceosome
61	460789	Heterogeneous nuclear ribonucleoprotein K	51,325	5.13	88	12	2.0	HNRNPK, HNRPK	Cytoplasm, Nucleus, Spliceosome
		Calcium ion binding protein							
62	4502101	Annexin A1	38,918	6.57	124	26	3.9	ANXA1, ANX1, LPC1	Cytoplasm, Nucleus, Cell membrane
63	56967118	Annexin A2	36,634	8.32	210	13	2.1	ANXA2	Basement membrane, Extracellular matrix, Secreted
		Signal transduction							
64	5803225	14-3-3 protein epsilon	29,326	4.63	136	26	2.5	YWHAE	Cytoplasm
65	4507949	14-3-3 protein beta/alpha	28,179	4.76	315	23	2.2	YWHAB	Cytoplasm
		Elongation factor							
66	704416	Elongation factor Tu	49,851	7.70	229	19	3.9	TUFM	Mitochondrion
		Proteasome regulatory							
67	4506221	26S proteasome non-ATPase regulatory subunit 12	53,270	7.53	127	6	3.3	PSMD12	Proteasome. Nucleus, Cytoplasm

Table 1. Continued

NO.	GI number	Functional category and Protein Name	Mr	pI	Score	%Coverage	Ratio	GeneID	Cellular component
		GTPase activation							
68	285975	Human rab GDI	51,088	5.94	347	15	3.3	RABGDIB	Cytoplasm
		Chromatin regulator							
69	20070220	Protein arginine N-methyltransferase 5	73,322	5.88	43	1	3.2	PRMT5	Cytoplasm, Nucleus
		Glycan metabolism							
70	48255891	Protein kinase C substrate 80K-H isoform 2	60,110	4.34	51	8	2.6	PRKCSH	Endoplasmic reticulum
		Protein disulfide isomerase							
71	20070125	Prolyl 4-hydroxylase, beta polypeptide	57,480	4.76	586	24	2.6	P4HB	Endoplasmic reticulum
		Protease inhibitor							
72	62898301	Serine proteinase inhibitor	42,857	5.90	167	9	2.3	SERPIN	Secreted

Functional studies of protein expression in cholangiocarcinoma cells cultured in matrix gel

The identified proteins that displayed significant changes in expression levels were analyzed the functional protein association networks by SIRING database to predict protein interactions (Figure 10). Moreover, they were classified using a UniProtKB search for protein functional analysis in the species *Homo sapiens* (human). Based on the search 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 (Figure 11). Both contact with the extracellular matrix (ECM) and the remodeling of the actin cytoskeleton can drive cancer cell motility and promote invasion. L-plastin is the one of actin-binding proteins that displayed 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 L-plastin expression was identified in RMCCA1 cells cultured in matrix gel (Figure 12). In addition, upregulation of pyruvate kinase M2 (PKM2), a key protein in the glycolysis pathway, in RMCCA1 cultured in matrix gel was also demonstrated (Figure 13).

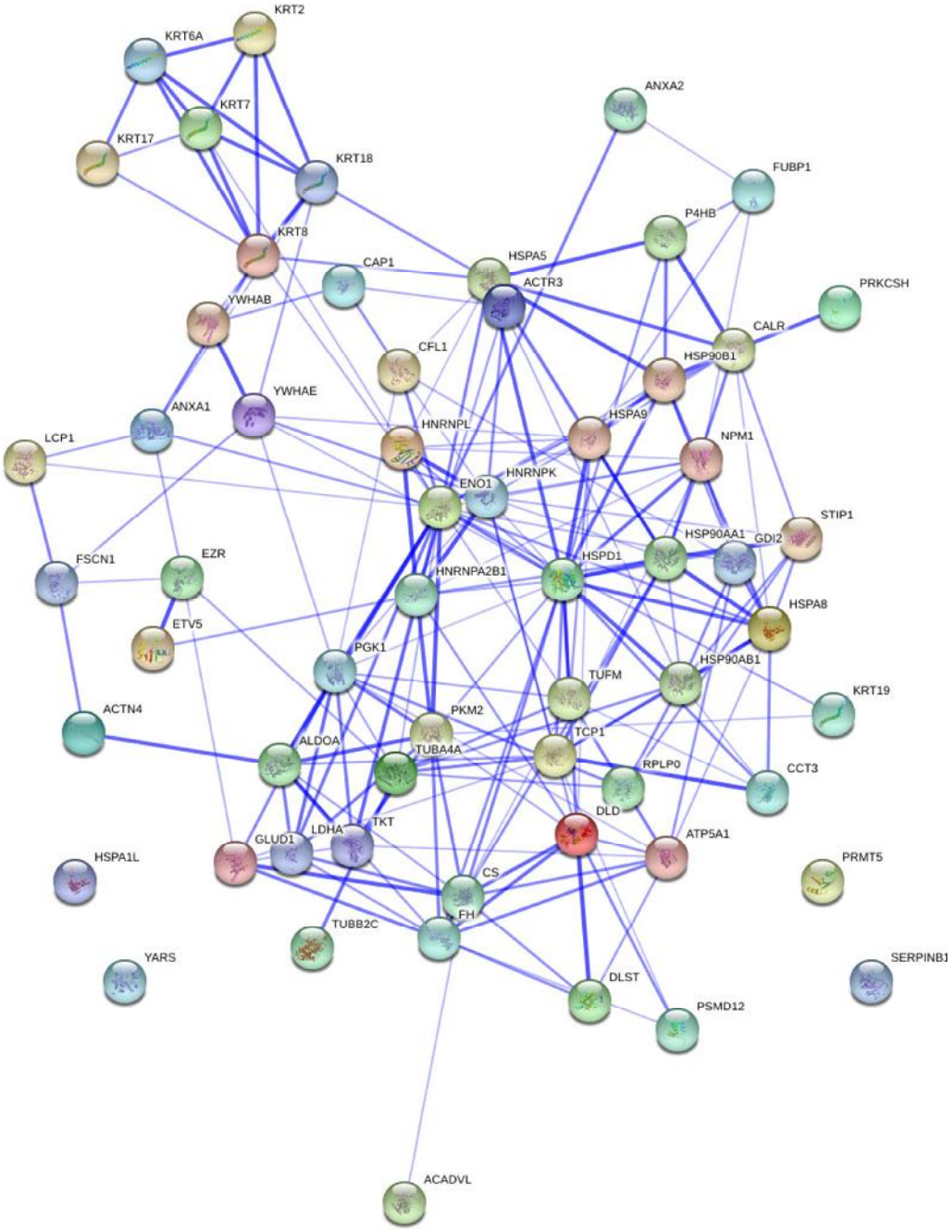


Figure 10. Functional protein association networks predicted protein interactions by STRING database. The network of interactions between proteins is generally represented as an interaction graph, where nodes represent proteins and edges represent pairwise interactions.

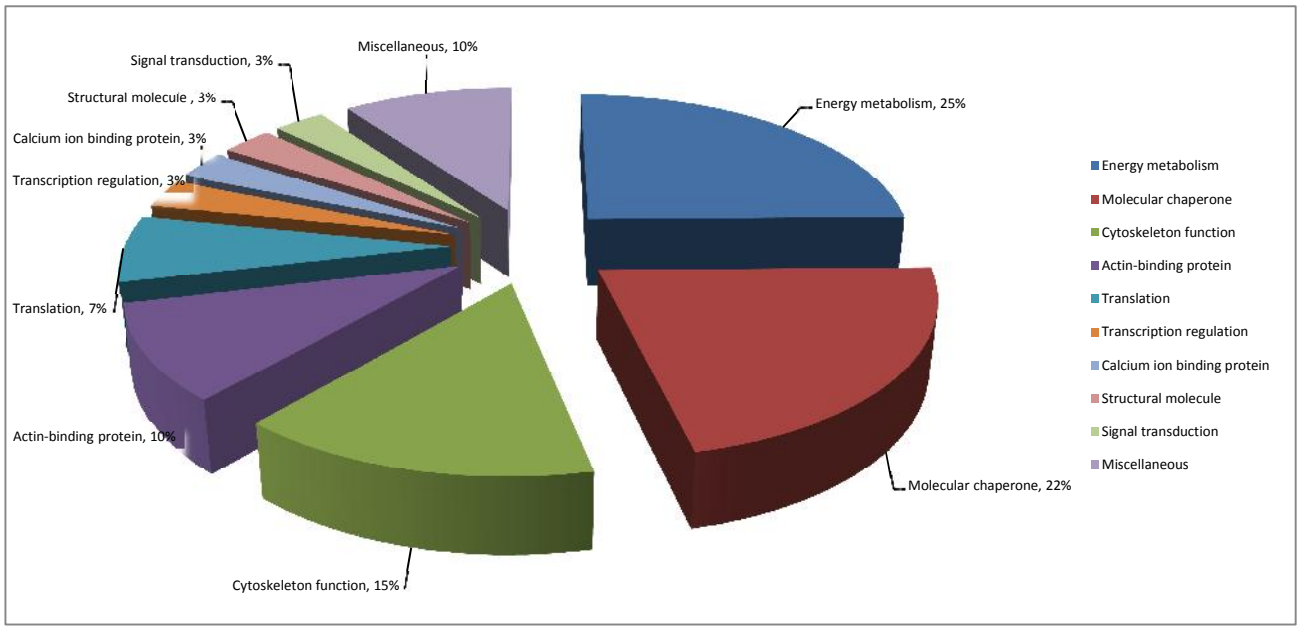


Figure 11. Functional classification of the differentially expressed proteins in cholangiocarcinoma cells cultured with matrix gel. The altered levels of protein expression were identified by mass spectrometric analysis (see Table 1) and categorized according to protein function. Note that the spots with the same identities were counted as only one spot, and each number represents the percentage among the total number of proteins identified.

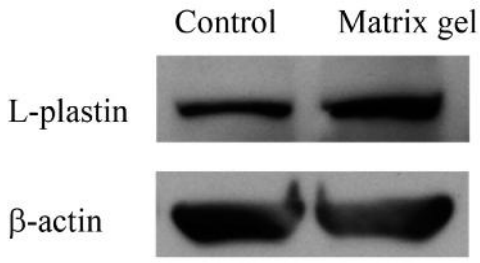


Figure 12. The expression levels of L-plastin in RMCCA1 cells following culture in matrix gel for 24 hr were determined by western blot analysis. Control was the cells that were cultured on uncoated plates. β-actin was used as a loading control.

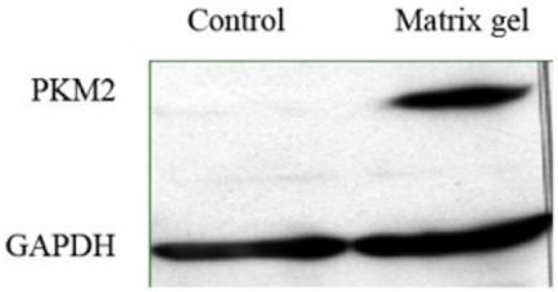


Figure 13. The expression levels of PKM2 in RMCCA1 cells following culture in matrix gel for 24 hr were determined by western blot analysis. Control was the cells that were cultured on uncoated plates. GAPDH was used as a loading control.

Effect of L-plastin on cholangiocarcinoma cell invasion

To demonstrate whether the expression of L-plastin is associated with cholangiocarcinoma cell invasion, we knocked down the expression of L-plastin using L-plastin shRNA. The western blot (Figure 14) and immunofluorescence studies (Figure 15) demonstrated that L-plastin was significantly downregulated after transfecting the RMCCA1 cells with L-plastin shRNA. Moreover, the invasion assay showed that the number of cancer cell invasion events was significantly decreased with the L-plastin shRNA cells compared with those treated with the control dsRNA ($P<0.001$) (Figure 16).

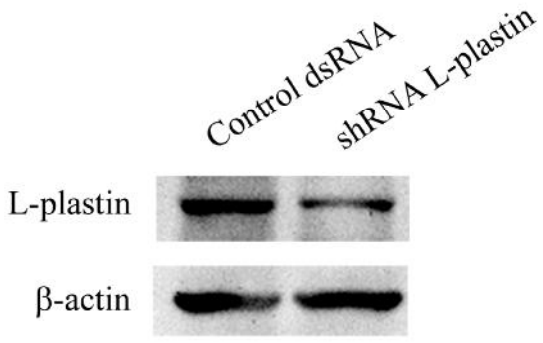


Figure 14. The western blot analysis after knocked down the expression of L-plastin using L-plastin shRNA. The expression levels of L-plastin and β-actin in RMCCA1 cells transfected with either control dsRNA or L-plastin shRNA were determined by western blot. Lane 1 represents protein extracted from RMCCA1 cells treated with control dsRNA, and Lane 2 represents protein extracted from RMCCA1 cells treated with L-plastin shRNA.

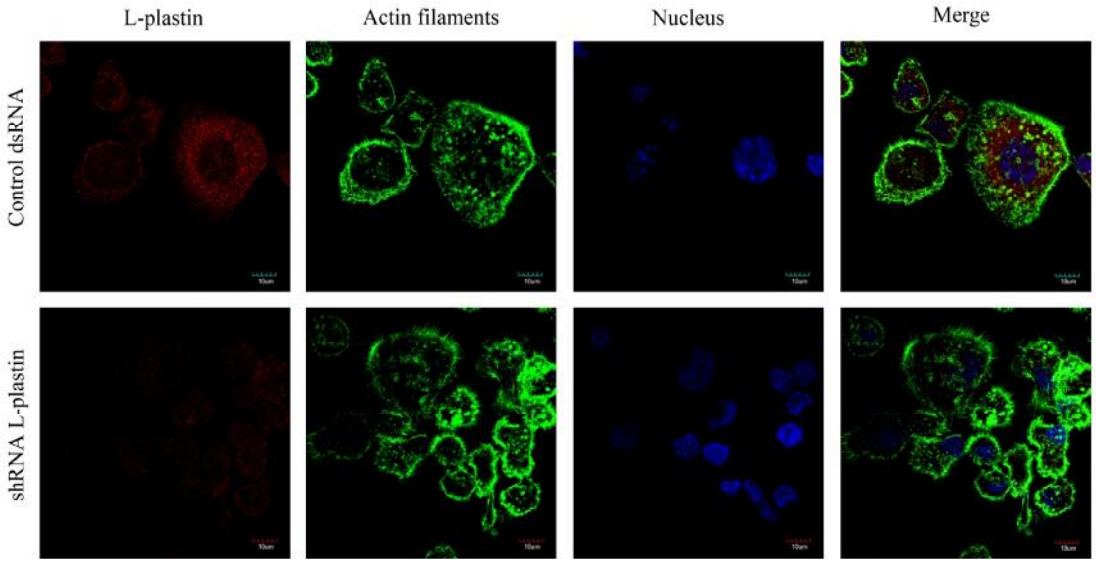


Figure 15. Immunofluorescence detection by confocal microscopy. The cells were transfected with either control dsRNA or L-plastin shRNA. The cells were then triple-stained with monoclonal L-plastin antibody (red), phalloidin (green, to reveal filamentous actin) or TOPO3 (blue, to reveal the nucleus). The scale bar indicates 10 μm.

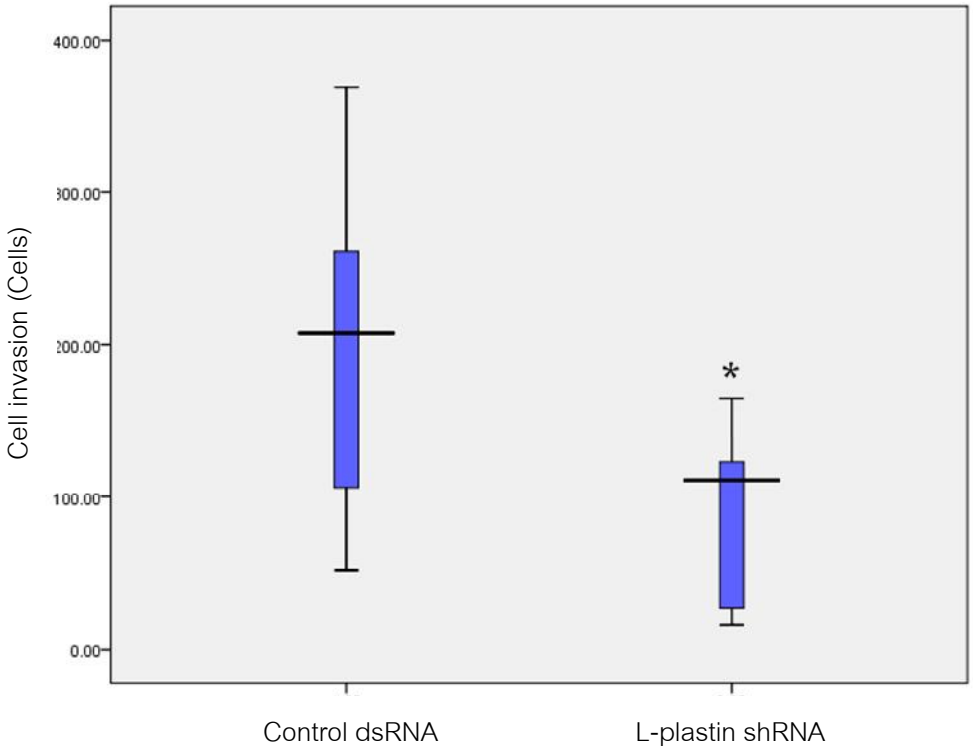


Figure 16. Box plots comparing the numbers of cholangiocarcinoma cell invasion events in cholangiocarcinoma cells treated with the control (dsRNA) and L-plastin shRNA (*; ANOVA; $P<0.001$ compared with the control).

Effect of PKM2 on cholangiocarcinoma cell invasion

To demonstrate whether the expression of PKM2 is associated with cholangiocarcinoma cell invasion, we knocked down the expression of PKM2 using PKM2 shRNA. The invasion assay showed that the number of cancer cell invasion events was significantly decreased with the PKM2 shRNA cells compared with those treated with the control dsRNA (P<0.001) (Figure 17).

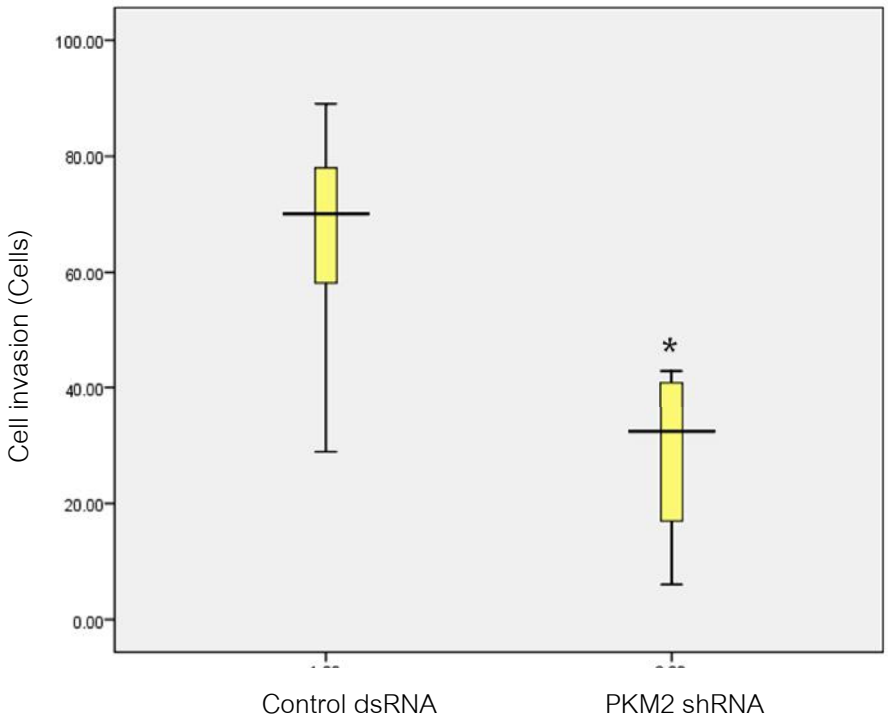


Figure 17. Box plots comparing the numbers of cholangiocarcinoma cell invasion events in cholangiocarcinoma cells treated with the control (dsRNA) and PKM2 shRNA (*; ANOVA; P<0.001 compared with the control).

CHAPTER V

DISSCUSSION

We report for the first time that the extracellular matrix (ECM) plays a major role in the regulation of cholangiocarcinoma cell invasion. For Matrix metalloproteinase (MMP) study, MMPs are involved in several pathological processes including cancers, inflammation and arthritis. Among the MMPs, MMP-9 and MMP-2 has been shown to be involved in breakdown of ECM in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis, intracerebral hemorrhage and metastasis. MMP-9 and MMP-2 secreted was detected in experiment of gelatin zymography. Only MMP-9 was shown the very high of expression in non-coated plates for incubation time at 24 hr. In Matrigel coated plates found less maybe because MMPs were trapped in the matrigel. So we cannot detect the expression in the supernatants.

Based on 2D electrophoresis results, we identified 72 proteins of unique 60 proteins that were upregulated when cholangiocarcinoma cells were cultured in matrix gel for 24 hr. Functional classification of the differentially expressed proteins in cholangiocarcinoma cells cultured with matrix gel was searched by UniProtKB for protein functional analysis. The percentages of each protein group among the total number of identified proteins, the high number (%) belong to the energy metabolism, molecular chaperoning, cytoskeleton functions and actin-binding proteins. L-plastin, a major F-actin-bundling protein of leucocytes and epithelial- or mesenchymal-derived cancer cells, was significantly upregulated in matrix-gel-coated plates compare with uncoated plates. The results were confirmed by western blotting, as L-plastin displayed higher expression levels in RMCCA1 cells cultured in matrix-coated plates. 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 [34]. In addition, L-plastin has also been detected in solid tumors of epithelial and mesenchymal origin and has been suggested

to play a role in cancer cell invasion [35]. 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 shRNA. From functional analysis L-plastin related proteins also increase together in systematic.

Confirming recent prior studies, we observed that L-plastin is located in the nuclei and cytoplasm of cancer cells [34, 47]. The functional relevance of the nucleocytoplasmic shuttling of L-plastin currently remains unclear. L-plastin may be involved in the regulation of nuclear actin, which is an essential component of the pre-initiation complex and cooperates with polymerases I, II and III in the regulation of gene expression [48]. The formation of protrusive structures is driven by spatially and temporally regulated actin polymerization at the leading edge of the cell [49]. Further studies should be performed to elucidate the involvement of L-plastin localization in cholangiocarcinoma cells. We found that L-plastin expression was observed mainly in cholangiocarcinoma cell, which are in the morphology of mesenchymal-like cells. These findings suggested that L-plastin expression may be related with epithelium-mesenchymal transition (EMT) of cholangiocarcinoma cell. Phosphorylation of L-plastin has been reported that it might function as a regulatory switch in the assembly of actin-rich structures involved in cell migration and signaling. Its phosphorylation shifts the protein from a low-activity to a high-activity state and might contribute to the regulation of the migratory behavior of cells in a 3D-space [33].

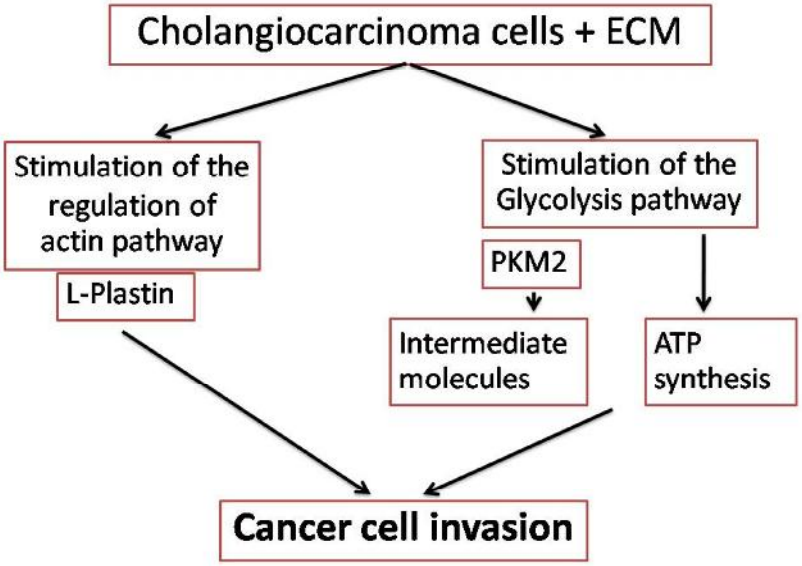
We demonstrated that cholangiocarcinoma cells cultured in matrix gel induce up-regulation of the several proteins associated with glycolysis pathways especially pyruvate kinase M2 (PKM2). PKM2 is involved in energy metabolism and highest over expression within this group. Pyruvate kinase catalyzes the last step within the glycolytic sequence, the dephosphorylation of phosphoenolpyruvate to pyruvate and is responsible for net energy production within the glycolytic pathway. Depending upon the different metabolic functions of the tissues, different isoenzymes of pyruvate kinase are expressed. The dimeric form of the pyruvate kinase isoenzyme type M2 (PKM2) is the predominant pyruvate kinase isoform in proliferating cells. Then, it will be a key

enzyme in tumor metabolism which found that it is up-regulated in many human tumors [41]. When PKM2 is mainly in the less active dimeric form, which is the case in cancer cells, all glycolytic intermediates above pyruvate kinase accumulate and are channeled into synthetic processes which branch off from glycolytic intermediates, such as nucleic acid-, phospholipid- and amino acid synthesis [43, 50, 51]. Therefore, we suggested that ECM induces cholangiocarcinoma cell synthesis the intermediates molecules for their invasiveness property (Figure 18). We suggested that future treatment of cholangiocarcinoma may be involved in the controlling of metabolism of cancer cells.

Functional protein association networks predicted protein interactions by STRING database were investigated. The interactions include direct (physical) and indirect (functional) associations; they are derived from four sources: Genomic Context, High-throughput Experiments, Conserved Coexpression and Previous Knowledge. The interactome represented as a graph where nodes correspond with proteins and edges with pairwise interactions [52]. Protein-protein interactions play major roles in the cell: transient protein interactions are often involved in post-translational control of protein activity. Protein interactions are crucial components of all cellular processes. From this result, L-plastin (LCP1) predicted to have the direct interaction to Fascin1 (FSCN1) and Annexin A1 (ANXA1). Moreover, we found the up-regulation of Fascin1 and Annexin A1 from gel-based proteomics study. Fascin1 is an actin-binding protein that has evidences overexpressed in many kinds of tumors and associated with lymph nodes metastasis and TNM staging. Up-regulated Fascin1 in non-small cell lung cancer promotes the migration and invasiveness, but not proliferation [53]. Annexin A1, a mediator of the anti-inflammatory action of glucocorticoids, is important in cancer development and progression [54]. Both Fascin1 and Annexin A1 have the interaction to Ezrin (EZR), which has been identified as the key regulatory molecules in cell motility, invasion and metastasis. For PKM2, it has interaction to many proteins mainly the proteins in glycolytic pathway such as Phosphoglycerate kinase 1 (PGK1), Aldolase A (ALDOA) and Enolase (ENO1).

In conclusion, attachment to the ECM promotes cholangiocarcinoma cell progression by inducing L-plastin expression and increase synthesis of glycolytic intermediates. Understanding these mechanisms may help to identify a novel molecular target for the development of an effective therapy for cholangiocarcinoma patients.

Figure 18. Diagram demonstrates the effects of ECM on the cholangiocarcinoma cell invasion.



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APPENDIX

APPENDIX

1. **Sample preparation solution (with urea and thiourea) for 2-D electrophoresis**

7 M Urea (FW 60.06)	10.5	g
2 M Thiourea (FW 76.12)	3.8	g
4% CHAPS	1.0	g
60 mM DTT (FW 154.2)	231	mg
Deionized water	to 25	ml

2. **Thiourea rehydration stock solution**

7 M Urea	10.5	g
2 M Thiourea	3.8	g
4% CHAPS	1.0	g
60 mM DTT	231	mg
IPG Buffer	125	µl
1% Bromophenol blue	50	µl
Deionized water	to 25	ml

3. **1% Bromophenol blue stock solution**

Bromophenol blue	10	mg
Tris-base	6	mg
Deionized water	to 1 ml	

4. **SDS equilibration buffer solution**

6 M Urea	72.1 g
1.5 M Tris-HCl, pH 8.8	10 ml
Glycerol (87% w/w)	69 ml
2% Sodium dodecyl sulfate, SDS (FW 288.38)	4.0 g
1% Bromophenol blue	400 µl
Deionized water	to 200 ml

5. **1.5 M Tris-HCl, pH 8.8**

Tris base (FW 121.1)	181.7 g
Deionized water	750 ml
HCl	adjust to pH 8.8
Deionized water	to 1 L

6. **10% SDS solution**

SDS	5 g
Deionized water	to 50 ml

Mix the solution and store at room temperature.

7. **10% ammonium persulfate solution**

Ammonium persulfate (FW 228.20)	0.1 g
Deionized water	to 1 ml

8. **SDS electrophoresis buffer**

25 mM Tris base	30.3	g
192 mM Glycine (FW 75.07)	144.0	g
0.1% SDS	10.0	g
Deionized water	to 10 L	

The pH of this solution should not be adjusted.

9. **Agarose sealing solution**

SDS electrophoresis buffer	100	ml
0.5% Agarose	0.5	g
1% Bromophenol blue	200	µl

10. **Colloidal Coomassie Blue G-250**

Coomassie Blue G-250	0.5	g
Ammonium sulfate (FW 132.1)	50	g
Phosphoric acid 85% (w/w)	6	ml
Deionized water	to 500 ml	
Methanol	125	ml

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