รายงานวิจัยฉบับสมบูรณ์

โครงการ
ตัวขนส่งกรดอะมิโนระบบแอล: เป้าหมายสำหรับการรักษาและการพยากรณ์ความรุนแรงของโรคมะเร็งท่อน้ำดี

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ธันวาคม 2559

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

และมหาวิทยาลัยมหิดล

Abstract

The highest incidence of cholangiocarcinoma (CCA) has been reported in the northeastern region of Thailand. Most patients are diagnosed at an advanced stage of disease in which the current medical treatments are ineffective. Therefore, the survival rate of CCA patients is relative poor. Thus, the discovery of new prognostic markers as well as the therapeutic targets are urgently needed. Previous studies have been reported that LAT1 and its associating protein 4F2hc were upregulated and associated with poor prognosis in various human cancers, suggesting their prognostic values in tumors. However, the information on LAT1 and 4F2hc expressions in Thai CCA tissues as well as their roles in CCA are limited. We, therefore, evaluated the prognostic significance of LAT1 and 4F2hc in 127 Thai ICC patients by using immunohistochemistry analysis. We found that LAT1 expression pattern was similar to that of 4F2hc. Interestingly, co-expression of LAT1 together with 4F2hc was significantly correlated with various clinicopathological factors compared to either the expression LAT1 or 4F2hc. Moreover, co-expression of LAT1 and 4F2hc was significantly related with shorter survival time of patients. However, from multivariate analysis, only TNM stage exhibited independent prognostic value. Therefore, to use LAT1 and 4F2hc as prognostic markers for CCA patient, the expression of LAT1 and 4F2hc together with other clinicopathological parameters are suggested. Roles of LAT1 was examined by using lentiviral shRNA mediating LAT1 knockdown in KKU-M213 cells, a cell line-derived from Thai CCA patient. Stable LAT1 knockdown suppressed the proliferative indefinitely properties, but did not alter cell proliferation as demonstrated by clonogenic assay and BrdU staining, respectively. In consistent, transient silencing LAT1 had no effect on cell proliferation but significantly suppressed cell invasion and migration. Most interestingly, LAT1 knockdown resulted in reduction the expression of its associating protein, 4F2hc, however, the expression of 4F2hc associating proteins, xCT, GLUT1 and β1-integrin was not altered. In addition, the expression of the negative 4F2hc regulator gene, miRNA-7 was significantly increased in transient LAT1 knockdown CCA cells. Lastly, the phosphorylation levels of Akt, p70S6K and ERK1/2 were inhibited in LAT1 knockdown KKU-M213 cells. Taken together, our results indicate that suppression of CCA cell survival and motility may be partly through the miRNA-7-related signaling pathways. Based on this finding, LAT1 and 4F2hc has a potential to be develop as prognostic biomarkers and therapeutic targets for CCA.

บทคัดย่อ

มะเร็งท่อน้ำดี (Cholangiocarcinoma) เป็นปัญหาสาธารณสุขที่สำคัญ ซึ่งอุบัติการณ์ของโรคมะเร็งชนิดนี้พบมากที่สุดในภาคตะวันออกเฉียงเหนือของประเทศไทย ส่วนใหญ่คนไข้จะถูกวินิจฉัยว่าเป็นมะเร็งชนิดนี้เมื่อโรคอยู่ในระยะที่รุนแรง ซึ่งจะดื้อต่อรักษาด้วยยาฆ่ามะเร็ง ทำให้ผู้ป่วยมีอัตราการรอดชีวิตต่ำ การค้นคว้าหาตัวบ่งชี้ในการพยากรณ์โรคและเป้าหมายใหม่สำหรับการรักษาจึงมีความสำคัญและจำเป็นอย่างยิ่ง ผลการวิจัยที่ผ่านมาพบว่าในเซลล์มะเร็งหลายชนิดมีระดับการแสดงออกของโปรตีนตัวขนส่งกรดอะมิโนแอลเอที 1 (LAT1) และโปรตีนที่ทำงานร่วม 4F2hc สูง ระดับการแสดงออกของ LAT1 และ 4F2hc ที่สูงจะมีความสัมพันธ์กับการพยากรณ์โรคที่ไม่ดี แสดงให้เห็นถึงความเป็นไปได้ที่จะใช้ระดับการแสดงออกของ LAT1 และ 4F2hc เป็นตัวพยากรณ์โรคมะเร็ง แต่ปัจจุบันยังขาดข้อมูลเกี่ยวกับการแสดงออกของโปรตีนทั้งตัวนี้ในชิ้นเนื้อมะเร็งท่อน้ำดีของคนไทย ตลอดจนบทบาทและกลไกที่เกี่ยวข้องในการดำเนินโรคของมะเร็งท่อน้ำดี จากการศึกษาการแสดงออกของโปรตีน LAT1 และ 4F2hc ในชิ้นเนื้อของมะเร็งท่อน้ำดีชนิด intrahepatic cholangiocarcinoma (ICC) จากคนไข้คนไทยจำนวน 127 ราย ด้วยวิธี immunohistochemistry พบว่าระดับการแสดงออกของ LAT1 มีความสัมพันธ์กับระดับการแสดงออกของ 4F2hc การแสดงออกร่วมกันของ LAT1 และ 4F2hc มีความสัมพันธ์กับลักษณะทางคลินิกมากกว่าการแสดงออกของ LAT1 หรือ 4F2hc เพียงอย่างเดียว ที่สำคัญการแสดงออกร่วมกันของทั้ง LAT1 และ 4F2hc มีความสัมพันธ์กับระยะเวลาการมีชีวิตรอดที่สั้นของคนไข้ จากการวิเคราะห์แบบ multivariate analysis พบว่าระดับการแสดงออกของ LAT1 และ 4F2hc เพียงอย่างเดียวไม่สามารถใช้เป็นตัวพยากรณ์โรคมะเร็งท่อน้ำดีได้ จึงเสนอแนะให้ใช้ร่วมกับปัจจัยทางคลินิกอื่น บทบาทของ LAT1 ต่อการดำเนินโรคของเซลล์มะเร็งท่อน้ำดีศึกษาโดยลดการแสดงออกของ LAT1 ในเซลล์มะเร็งท่อน้ำดีชนิด KKU-M213 ซึ่งเป็นเซลล์มะเร็งท่อน้ำดีที่พัฒนามาจากคนไข้คนไทย การลดการแสดงออกของ LAT1 แบบถาวรยับยั้งการเจริญเติบโตอย่างไร้ขีดจำกัดหรือความสามารถในการอยู่รอดของเซลล์มะเร็งท่อน้ำดี แต่ไม่มีผลต่อการเพิ่มจำนวนของเซลล์สอดคล้องกับผลการทดลองจากการลดการแสดงออกของ LAT1 แบบชั่วคราว ซึ่งไม่มีผลต่อการเพิ่มจำนวนเซลล์ แต่มีผลยับยั้งการบุกรุกและการแพร่กระจายของเซลล์ KKU-M213 ที่สำคัญยังพบว่าระดับการแสดงออกของ 4F2hc ลดลงในเซลล์ที่ลดการแสดงออกของ LAT1 แต่ไม่พบการเปลี่ยนแปลงของการแสดงออกของโปรตีนที่อาศัยการทำงานร่วมกับโปรตีน 4F2hc คือ xCT, GLUT1 และ β1-integrin นอกจากนี้ยังพบว่าการลดการแสดงออกของ 4F2hc พบร่วมกับการเพิ่มการแสดงออกของ miRNA-7 ซึ่งปกติทำหน้าที่ลดการแสดงออกของ 4F2hc การลดการแสดงออกของ LAT1 ลดการทำงานของ Akt, p70S6K และ ERK1/2 จากผลการศึกษาทั้งหมดอาจกล่าวได้ว่า LAT1 ยับยั้งคุณสมบัติของเซลล์มะเร็งท่อน้ำดีในการเจริญเติบโตอย่างไร้ขีดจำกัด การบุกรุกและแพร่กระจาย อย่างน้อยอาจผ่านการยับยั้งการทำงานของกลไกวิถีที่เกี่ยวข้องกับ miRNA-7 ดังนั้นการทดลองครั้งนี้แสดงให้เห็นถึงความสามารถในการใช้โปรตีนตัวขนส่งกรดอะมิโน LAT1 เป็นตัวพยากรณ์และเป้าหมายของการรักษาโรคมะเร็งท่อน้ำดี

Executive Summary

Cancer is the leading cause of death worldwide accounting for 7.6 million deaths (13% of all deaths) in 2008. Liver cancer was the third most common cancer death. About 70% of all cancer deaths occurred in low- and middle-income countries (GLOBOCAN 2008). In Thailand, liver and bile duct cancer deaths were ranked in top five in both male and female for the year of 2004. Liver cancers are divided into two major subtypes with distinguishing histological characteristics and origins which are hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA). CCA is accounted for more than 70% of primary liver cancer where the highest prevalence is reported to be in the northeastern part of Thailand. The major risk factor of CCA in this region has been shown to be associated with infection of liver fluke, *Opisthorchis viverrini*. Due to its clinically silent, most of CCA patients are diagnosed at the advanced stages which are not amenable to surgical resection. In addition, the current available chemotherapeutic drugs are ineffective, therefore, the prognosis of CCA patients is poor and the patients die within a year after diagnosis. Thus, the objectives of current proposal are to identify new prognostic markers as well as therapeutic targets to improve treatment outcome for CCA patients. Previous studies have been reported that LAT1 and its associating protein 4F2hc were upregulated and associated with poor prognosis in various human cancers, suggesting their prognostic values in tumors. However, the expressions of LAT1 and 4F2hc in Thai CCA tissues as well as their roles in CCA are limited. In this project, we examined the expression of LAT1 and 4F2hc in 127 Thai ICC patients by using immunohistochemistry analysis. We found that co-expression of LAT1 together with 4F2hc was significantly correlated with various clinicopathological factors and shorter survival time of patients compared to either the expression LAT1 or 4F2hc alone. However, from multivariate analysis, only TNM stage exhibited independent prognostic value, thus, the expressions of LAT1 and 4F2hc are suggested to use together with other clinicpathological parameters as prognostic markers for CCA patients. Although, we have completed this objective, however, the identical results have been recently reported. Therefore, to publish this part of our research work, together with LAT1/4F2hc expression we are trying to investigate the expression of other potential markers in these CCA tissue samples. Moreover, we have recently found the expression of 4F2hc in exosomes, the small vesicles secreted from KKU-M213 cells but not from normal cholangiocyte cells. This finding also highlights the potential use of 4F2hc in circulating exosomes as prognostic biomarker for CCA and the paper has been published. Next, roles of LAT1/4F2hc in CCA carcinogenesis were examined by using lentiviral shRNA mediating LAT1 knockdown in KKU-M213 cells, a cell line-derived from Thai CCA patient. We have found that LAT1

silencing suppressed KKU-M213 cell survival and motility. LAT1 knockdown resulted in reduction of 4F2hc expression however, the expressions of 4F2hc associating proteins were not altered. Most interestingly, the expression of the negative 4F2hc regulator gene, miRNA-7 was significantly increased in LAT1 knockdown CCA cells, highlighting a new molecular mechanisms underlying CCA carcinogenesis-mediated by LAT1. This part of our research works have been completed and published. It would be interesting to further investigate roles of this non-coding small RNA in CCA carcinogenesis.

Introduction to the research problem and its significance:

Cancer is the leading cause of death worldwide and accounting for 7.6 million deaths (13% of all deaths) in 2008. Liver cancer was the third most common cancer death. About 70% of all cancer deaths occurred in low- and middle-income countries (GLOBOCAN 2008). In Thailand, liver and bile duct cancer deaths were ranked in top five in both male and female for the year 2004 (Review in (2)). Liver cancers are divided into two major subtypes with distinguishing histological characteristics and origins, hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA). Although CCA is a rare tumor in many parts of the world, however it is highly prevalent in the northeastern part of Thailand where CCA accounted for more than 70% of primary liver cancer (Sripa et al., 2012; Sripa and Pairojkul, 2008). Here, the major risk factor of cholangiocarcinoma has been shown to be associated with infection of liver fluke, *Opisthorchis viverrini* from poor sanitation practice (Sripa and Pairojkul, 2008). CCA is classified to intrahepatic, perihilar and distal extrahepatic subtypes according to its anatomical landmark (Blechacz et al., 2011). Intrahepatic and extrahepatic CCA accounted for 40% and 60%, respectively of all cases indentified in countries where *Opisthorchis viverrini* is endemic, however most cases indentified in Khon Kaen province are intrahepatic CCA (Sripa and Pairojkul, 2008). Patients usually present at late stage which is not amenable to surgical therapies because CCA is clinically silent and the symptoms usually develop only at an advanced stage (Blechacz et al., 2011). Therefore, the prognosis of these CCA patients is poor, and people die within a year after diagnosis. Surgical resection is the only curative therapy for CCA, which unfortunately have no improvement with long-term survival (Aljiffry et al., 2009; Mosconi et al., 2009) and often not accessible for most of patients in developing countries, including Thailand. In addition, the chemotherapy is ineffective for cholangiocarcinoma (Blechacz and Gores, 2008). Therefore, discovery of new prognostic markers as well as therapeutic targets is an essential step in development of effective treatment strategies or drugs.

Literature review:

System L amino acid transporters

System L amino acid transporter serves as the major route for uptake of essential amino acids into the cell (Christensen, 1990). L-type amino acid transporter 1 (LAT1), the first isoform of

system L amino acid transporter isolated from C6 rat glioma cells is a 12-membrane-spaning protein, which mediates Na^+-independent amino acid exchange (Kanai et al., 1998). LAT1 forms a heterodimeric complex with the heavy chain of 4F2 antigen (4F2hc or CD98) that serves as a chaperone for recruitment of LAT1 to the plasma membrane (Fig. 1) (Kanai et al., 1998). LAT1 transport of large neutral amino acids can be inhibited by a system L-specific inhibitor 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH). It was expressed in brain, testis and placenta. Later on, the second isoform, L-type amino acid transporter 2 (LAT2) was identified and similar to LAT1, it forms a complex with CD98. Its transport activity was also inhibited by BCH. In contrast, LAT2 has a broad substrate specificity including all neutral amino acids except proline (Pineda et al., 1999; Segawa et al., 1999). In 2003, amino acid transporter system L2 was identified (Babu et al., 2003; Bodoy et al., 2005) and known as LAT3 and LAT4. Both are Na^+-independent plasma membrane transporters with low affinity for large neutral amino acids. For functional expression, there are not required 4F2hc. The amino acids uptake by LAT3 and LAT4 was inhibited by BCH consistent with the properties of system L (Babu et al., 2003; Bodoy et al., 2005). LAT4 was showed 57% identity to LAT3, however it has a different tissue distribution (Bodoy et al., 2005). LAT3 was highly expressed in liver and pancreas (Babu et al., 2003). However, LAT4 was predominantly expressed in human placenta, peripheral blood leukocyte and brain (Bodoy et al., 2005).

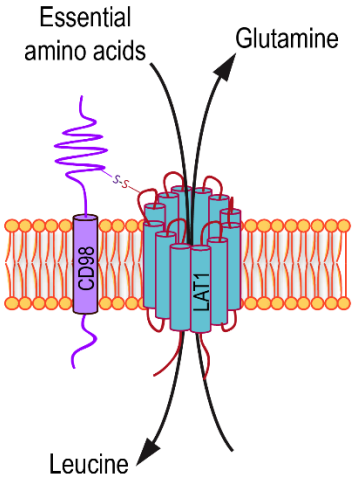

Figure 1. LAT1-4F2hc (CD98) heterodimeric amino acid transporter

Expression of system L amino acid transporters and 4F2hc in cancer

The upregulation of LAT1 and 4F2hc has been observed in several solid tumors such as brain, colon, lung, liver and skin (Fuchs and Bode, 2005), and in several cancer cell lines such as leukemia cells, lung small cell carcinoma cells (RERF-LC-MA), uterine cervical carcinoma cells (HeLa) and bladder carcinoma cells (T24) (Yanagida et al., 2001). Inhibition of LAT1 mediated amino acid uptake by BCH, a specific system L inhibitor, suppressed growth of ovarian cancer cells; SKOV3, IGROV1 (Fan et al., 2010), prostate cancer cells; LNCaP, PC-3 (Wang et al., 2011), human laryngeal squamous cell carcinoma; Hep-2 (Yamauchi et al., 2009), human oral epidermoid carcinoma cells; KB, and human osteogenic sarcoma cells; Saos2 (Kim et al., 2008). LAT1 induced ovarian cancer cell migration and mouse trophoblast stem cells invasion (Chrostowski et al., 2010; Kaji et al., 2010). However, the number of migrated cells was decreased when treated with BCH. Recent studies have demonstrated that high level expression of LAT1 and 4F2hc was associated with poor prognosis in various human cancers, such as pancreatic cancer (Kaira et al., 2012), stage 1 sequamous cell carcinoma of the lung (Kaira et al., 2009), primary astrocyte tumor (Nawashiro et al., 2006), and triple negative breast cancer (Furuya et al., 2012). These significant correlations of LAT1 and 4F2hc expression with clinicopathological features and patient survival suggest the prognostic value of LAT1 and 4F2hc in tumors. Role of these system L2 amino acid transporters in cancer progression is unclear. However, LAT3 was indentified from human hepatocarcinoma-derived cell line FLC4 (Babu et al., 2003). Knockdown of LAT3 in prostate cancer cell lines (LNCaP and PC-3) by shRNA resulted in a significant decrease in cell growth (Wang et al., 2011). LAT4, the second isoform of amino acid transporter system L2 expressed at high level compared to that of LAT1 in human colorectal adenocarcinoma cells (HT-29) and human head and neck squamous cell carcinoma (FaDu) (Haase et al., 2007) however role of LAT4 in these cancer cell lines is unknown. Collectively, LAT1 and 4F2hc is a promising pathological marker for the prediction of patient outcome and could be one of the molecular targets in cancer therapy.

Molecular signaling for LAT1 and 4F2hc in inhibition of cancer growth

Mammalian target of rapamycin or mTOR regulates cell growth and proliferation by phosphorylating its downstream targets p70S6K (the 70 kDa Ser/Thr kinase) and 4E-BP, and then

increases rate of protein synthesis, which influences cell growth (Fingar and Blenis, 2004). Amino acids are known to stimulate mTOR. Amino acid starvation, in particular leucine, results in a dephosphorylation of p70S6K and 4E-BP1, whereas re-addition of amino acids restores p70S6K and 4E-BP1 phosphorylation (Hay and Sonenberg, 2004). Among the substrates of LAT1, leucine is the most potent amino acid to activate p70S6K phosphorylation (Shigemitsu et al., 1999). Indeed, over-expression of LAT1 in *Xenopus* oocytes confers sensitivity of TOR pathway to extracellular amino acids (Christie et al., 2002). Recent study showed that downregulation of LAT1 and CD98 by siRNA inhibited mTOR activity in HeLa cells (Nicklin et al., 2009). However, the mechanism by which amino acids signal to mTOR has remained largely unknown.

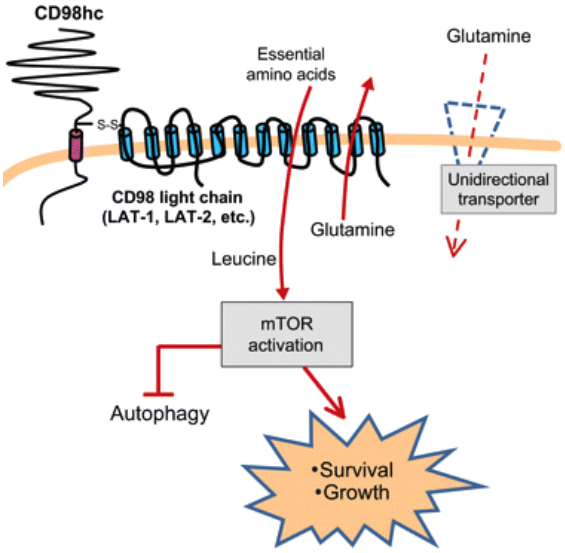

Figure 2. Activation of mTOR signaling pathway by LAT1 mediated amino acid uptake in cancer (Cantor and Ginsberg, 2012)

4F2hc or CD98 is a single membrane spanning protein, which is involved in a variety of cell activities. 4F2hc binds to LAT1, LAT2, y^+-LAT1, y^+-LAT2, Asc1 and xCT that serves as a chaperone for recruitment these amino acid transporters to the plasma membrane for functional expression (Verrey et al., 2004). Highly expression of 4F2hc in solid tumors is correlated with progressive and metastatic tumors (Furuya et al., 2012; Kaira et al., 2011; Kaira et al., 2009; Kaira et al., 2012; Kaira

et al., 2010), suggesting an important role of 4F2hc in tumorigenesis. Previous studies were also shown that inhibition of 4F2hc by anti-4F2hc antibodies suppressed human bladder cancer cell growth (Yagita et al., 1986), and overexpression of 4F2hc resulted in malignant transformation of murine fibroblast cells, BALB3T3 (Shishido et al., 2000) and tumorigenesis of colorectal cancer (Nguyen et al., 2012). Recently, it has been reported that 4F2hc contributes in malignant transformation by amplifying integrin signaling. Integrins sense the extracellular matrix (ECM) stiffness and that increased in ECM stiffness promotes tumor progression through focal adhesion kinase (FAK) and phosphatidylinositol 3-kinase (PI3K) pathways (Levental et al., 2009). Overexpression of 4F2hc induced cellular transformation through activation PI3K-FAK signaling and that required interaction between 4F2hc and β1 integrin (Fig. 3) (Henderson et al., 2004). Furthermore, it has been shown that deletion of 4F2hc impaired integrin signaling (Feral et al., 2005). Collectively, 4F2hc might control tumorigenesis by governing integrin signaling.

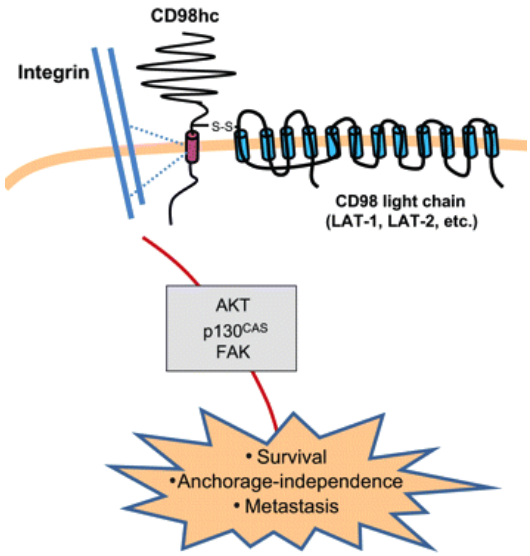

Figure 3. CD98-interin signaling in cancer (Cantor and Ginsberg, 2012)

xCT belongs to amino acid transporter system X_c^-, and functions as a Na^+-independent exchange system for cystine/glutamate. It required 4F2hc/CD98 for its functional expression at plasma membrane (Verrey et al., 2004). xCT is essential for uptake of cystine required for intracellular GSH synthesis that is important for maintaining intracellular redox balance in normal cells and cancer

cells (Verrey et al., 2004). It has been demonstrated that xCT is highly expressed in various malignant tumors, such as leukemia, lymphoma, and pancreatic cancer and exhibited low level of ROS (Fig. 4) (Huang et al., 2005; Lo et al., 2008). Disruption of xCT by RNAi and inhibitor in hepatocellular carcinoma cells resulted in an increase in intracellular reactive oxygen species (ROS) levels, and in turn led to autophagic cell death (Guo et al., 2011). Therefore, downregulation of xCT is proposed to be a promising strategy for cancer treatment.

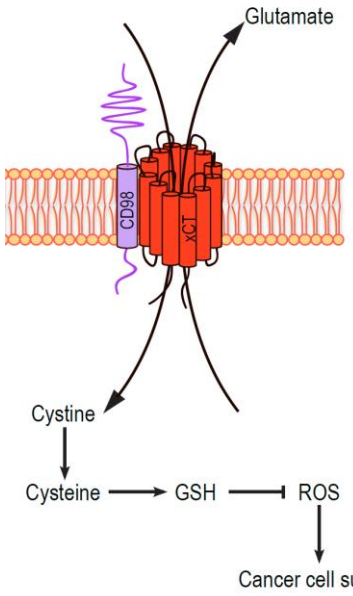

Figure 4. Role of xCT-CD98 amino acid transporter in cancer

In summary, based on the available experimental data to date, 4F2hc and its associating partners are the promising targets for cancer therapy. However, 4F2hc is a multifunctional protein, therefore targeting 4F2hc might cause an unwanted or unexpected negative effects. As mentioned above, LAT1 is predominantly expressed in tumor and we found for the first time that knockdown of LAT1 by RNAi surprisingly suppressed 4F2hc expression. Strategies aimed to disrupt LAT1 function/activity in current proposal may, thus, provide a therapeutic benefit for cholangiocarcinoma.

Objectives

1. Investigation of the correlation between LAT1 and 4F2hc expression with clinicopathological parameters of CCA

2. To investigate the role of LAT1 and its molecular mechanisms for inhibition of tumor progression in both *in vitro* and *in vivo* model

Results and Discussion

Objective 1. Investigation of the correlation between LAT1 and 4F2hc expression in CCA tissue specimens with its clinicopathological parameters

Background and Rationale:

High level expressions of LAT1 and 4F2hc in tumor tissues were associated with poor prognosis in various human cancers, such as pancreatic cancer, stage 1 sequamous cell carcinoma of the lung, primary astrocyte tumor, and triple negative breast cancer, suggesting the prognostic values of LAT1 and 4F2hc in tumors. However, the prognostic significance of LAT1 and 4F2hc in cholagiocarcinoma is unknown. Therefore, the objective of this study is to study the correlation between LAT1 and 4F2hc expressions with clinicopathologcal parameters of CCA.

Method:

The paraffin-embedded human CCA specimens (approximately 100-200 samples) was provided by Associate Professor Noppadol Larbcharoensub (Department of Pathology, Ramathibodi Hospital Mahidol University, Thailand). The tissue samples from 127 patients were selected from a cohort of 205 consecutive cases according to the availability of complete clinical data and tissue samples. All cases were confirmed pathologically as mass-forming, periductal infiltrating and intraductal-growth subtype of ICC and were diagnosed and treated at Faculty of Medicine Ramathibodi Hospital, Mahidol University between 2007 and 2011. Extrahepatic cholangiocarcinoma, adenocarcinoma of the gallbladder and combined hepatocellular-cholangiocarcinoma were excluded. Assessment of clinical stages was based on the American Joint Committee on Cancer (AJCC) system. Histopathological grades were evaluated according to World Health Organization (WHO) classifications. Patients were staged by physical examination, computed tomography or magnetic resonance imaging of the abdomen, complete blood counts, liver and renal function tests, chest X-ray and bone scintigraphy. All patients were followed-up every 3–6 months until death. The use of human materials was approved by the research ethics committee of the Faculty of Medicine Ramathibodi Hospital, Mahidol University (ID 10-54-18).

Paraffin-embedded tissues were cut at a thickness of 4 μm. Tissue sections were deparaffinized and treated with 3% hydrogen peroxide to inactivate endogenous peroxidases. The slides were blocked with 3% bovine serum albumin in PBS for 30 min and incubated with either rabbit polyclonal antibodies against LAT1 (SLC7A5 at a dilution of 1:100), CD98 (4F2hc at a dilution of 1:200) or Ki67 (polyclonal at a dilution of 1:100) for 1 hour at room temperature in a moist chamber. The sections were subsequently washed with PBS and incubated in horseradish peroxidase-conjugated antibody for 30 min according to the manufacturer's instructions. Immunoreactivity was visualized with diaminobenzidine substrate and nuclei were counterstained with haematoxylin. Normal human hepatic tissues and gallbladders were included as negative controls.

Results and Discussion:

Overexpression of amino acid transporter is crucial to supply the sufficient essential amino acids for tumor cells. Among them, upregulation of LAT1 and it associating protein, 4F2 heavy chain (4F2hc) have been reported in tumors and was associated with their malignant transformation, suggesting the prognostic significance of LAT1 and 4F2hc in these tumor. However, the expressions and prognostic values of LAT1 and 4F2hc in CCA tissue of Thai patients have not been investigated. In present study, the expressions of LAT1 and 4F2hc in Thai intrahepatic cholangiocarcinoma (ICC) tissues were assessed by immunohistochemistry. Clinical characteristics of tumor tissues are listed in Table 1. The median age of ICC patients is 59 years old ranging between 28 to 86 years old both male and female. Median survival time of the 127 patients was 7 months. Most of tissues are stage 2 to 4 (TNM and T stage). Tissue with lymph node (N1) and other organ (M1) metastasis are 66.9% and 37%, respectively. Therefore, the data obtained from this study may not be applicable to use as marker for early detection, however, it may be used as a prognosis biomarker for ICC. Moreover, tissues with well, moderately and poorly differentiated was induced in our present study.

The expression of LAT1 and 4F2hc is shown in figure 1. The localization of LAT1 and 4F2hc proteins were detected and localized predominantly on plasma membrane of CCA cells and classified as positive staining (Figure 4, A-B). However, the negative staining of LAT1 and 4F2hc proteins were also observed in some CCA tissues (Figure 4, C-D). The correlations between the expressions of LAT1 and 4F2hc with the clinicopathological parameters, including TNM stage, T stage, N stage, M stage, lymphovascular space invasion, grading, papillary pattern, the expression of Ki-67, serum tumor marker (AFP, CEA and CA19.9) and survival time are listed in Table 2. The positive staining for LAT1 and 4F2hc was 45% (58/127) and 54% (69/127), respectively. In addition,

the positive staining for both of LAT1 and 4F2hc was 39% (50/127). LAT1 expression was associated significantly with TNM stage (p=0.016), M stage (p=0.045) and CA19-9 level (p=0.040). 4F2hc expression was associated significantly with TNM stage (p=0.050), AFP level (p=0.038) and CA19-9 level (p=0.016). Neither LAT1 nor 4F2hc expression was associated significantly with other clinicopathological factors including age, gender, T stage, lymph node involvement, lymphovascular invasion, grade, papillary pattern, Ki67 expression, and CEA level. Interestingly, co-expression of LAT1 and 4F2hc was found to be correlated significantly with many parameters compared to either LAT1 or 4F2hc positive staining alone including higher TNM stage ($p < 0.001$), N stage ($p = 0.036$), M stage ($p = 0.002$), lymphovascular space invasion ($p = 0.041$), and CA 19-9 level ($p = 0.037$).

The correlation between survival time and the expression of LAT1, 4F2hc, LAT1+4F2hc was also determined using univariate analysis. We found that the expressions of either LAT1 or 4F2hc were not correlated with 1-year and 2-year survival rates. Interestingly, the positive staining of both LAT1 and 4F2hc was correlated significantly with shorter survival time ($p = 0.007$). The survival time for patients with and without LAT1+4F2hc expression were 8.74 ± 10.60 and 13.37 ± 14.07 months, respectively. The cumulative survival curves according to the significant prognostic variables are shown in figure 2. The patients with positive staining of LAT1 and 4F2hc alone showed only slightly shorter survival time compared to the negative staining cases (Figure 5, A and B). Interestingly, the patients with positive staining of both LAT1 and 4F2hc showed markedly shorter survival time (Figure 5C). A subsequent multivariate Cox regression analysis of significant prognostic determinants from the univariate analysis is shown in Table 3. Only TNM stage exhibited independent prognostic value (relative risk 2.110; 95% confidence interval = 1.035-4.300; $p = 0.040$). These results suggest that the expression of LAT1 and 4F2hc may not be used as independent prognostic marker for intrahepatic cholangiocarcinoma. Therefore, to determine the prognosis of Thai ICC patients, the information on expression levels of LAT1 and 4F2hc together with other clinicopathological parameters may be more appropriate. However, the expressions of LAT1 and 4F2hc significantly correlated with shorter 2-year survival time suggesting the important role of LAT1 and 4F2hc in carcinogenesis of ICC.

Table 1. Clinical characteristic of ICC patients.

Characteristic		No. of patients	Percentage
Age (year)			
	Mean	59.86	
	Median	59	
	Range	28-86	
Sex			
	Male	74	58.3
	Female	53	41.7
TNM stage			
	2	14	11
	3	66	52
	4	47	37
T stage			
	1	1	0.8
	2	31	24.4
	3	90	70.9
	4	5	3.9
N stage			
	0	42	33.1
	1	85	66.9
M stage			
	0	80	63
	1	47	37
Tumor size (cm) (n=110)			
	Mean	7.64	
	Median	7.4	
	Range	1.1-23.9	
Grade			
	Well	46	36.2
	Moderately	49	38.6
	Poorly	32	25.2
AFP (ng/ml) (n=111)			
	Median	3.75	
	Range	0.80-44639	
CEA (ng/ml) (n=107)			
	Median	4.91	
	Range	0.46-6144	
CA19-9 (U/ml) (n=116)			
	Median	169.15	
	Range	0-197016.80	
Overall survival (month)			
	Mean	11.54	
	Median	7.00	
	Range	1-63	

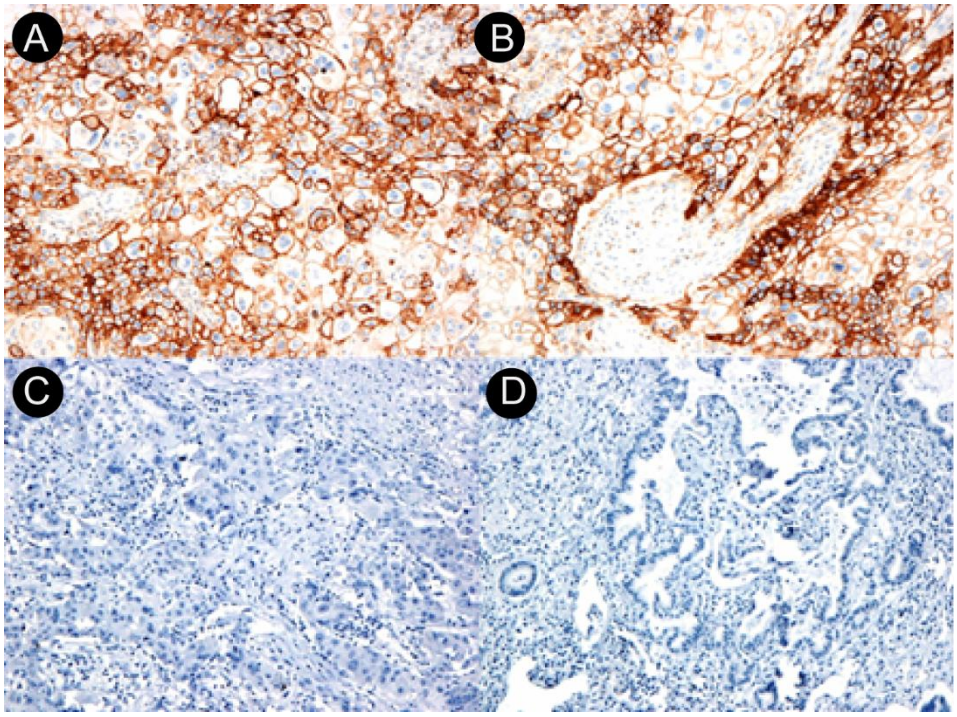

Figure 4. Immunohistochemistry of L-type amino acid transporter 1 (LAT1) and 4F2hc in intrahepatic cholangiocarcinoma. Tissue sections were stained with polyclonal antibodies against LAT1 (A, C) and 4F2hc (B, D). Immunostaining patterns in A and B are classified as positive staining. Immunostaining patterns in C and D are classified as negative staining.

Table 2. Univariate analysis between the expression of LAT1 and 4F2hc with clinicopathological parameters

Parameter		LAT1			4F2hc			LAT1 + 4F2hc		
		Positive	Negative	*p* -value	Positive	Negative	*p* -value	Positive	Negative	*p* -value
TNM stage										
	2	2	12	**0.016**	3	11	**0.05**	0	14	**<0.001**
	3	29	37		29	37		23	43	
	4	27	20		27	20		27	20	
T stage										
	1	1	0	0.646	1	0	0.634	1	0	0.469
	2	14	17		15	16		12	19	
	3	40	50		40	50		34	56	
	4	3	2		3	2		3	2	
N stage										
	0	15	27	0.132	17	25	0.353	11	31	**0.036**
	1	43	42		42	43		39	46	
M stage										
	0	31	49	**0.045**	32	48	0.067	23	57	**0.002**
	1	27	20		27	20		27	20	
LVSI										
	Negative	12	21	0.23	14	19	0.686	8	25	**0.041**
	Positive	46	48		45	49		42	52	
Grade										
	Well	25	21	0.335	25	21	0.392	22	24	0.301
	Moderat	20	29		20	29		18	31	
	Poorly	13	19		14	18		10	22	
Papillary pattern										
	Positive	7	8	>0.999	7	8	>0.999	6	9	>0.999
	Negative	51	61		52	60		44	68	
Ki67 (less than										
	<25%	24	40	0.076	26	38	0.215	20	44	0.071
	≥25%	34	29		33	30		30	33	
AFP (n = 111)										
	<7.02	36	42	0.835	41	37	**0.038**	34	44	0.137
	≥7.02	14	19		10	23		9	24	
CEA (n = 107)										
	<10	30	35	0.69	30	35	0.432	24	41	>0.999
	≥10	17	25		16	26		15	27	
CA19.9 (n =116)										
	<100	18	34	**0.04**	18	34	**0.016**	15	37	**0.037**
	≥100	35	29		37	27		31	33	
1-year survival										
	Alive	14	21	0.55	14	21	0.428	9	26	0.067
	Death	44	48		45	47		41	51	
2-year survival										
	Alive	6	12	0.313	6	12	0.309	3	15	**0.039**
	Death	52	57		53	56		47	62	

Abbreviation: LAT1, L-type amino acid transporter 1; LVSI, lymphovascular space invasion; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9. Bold values indicate a statistically significant difference.

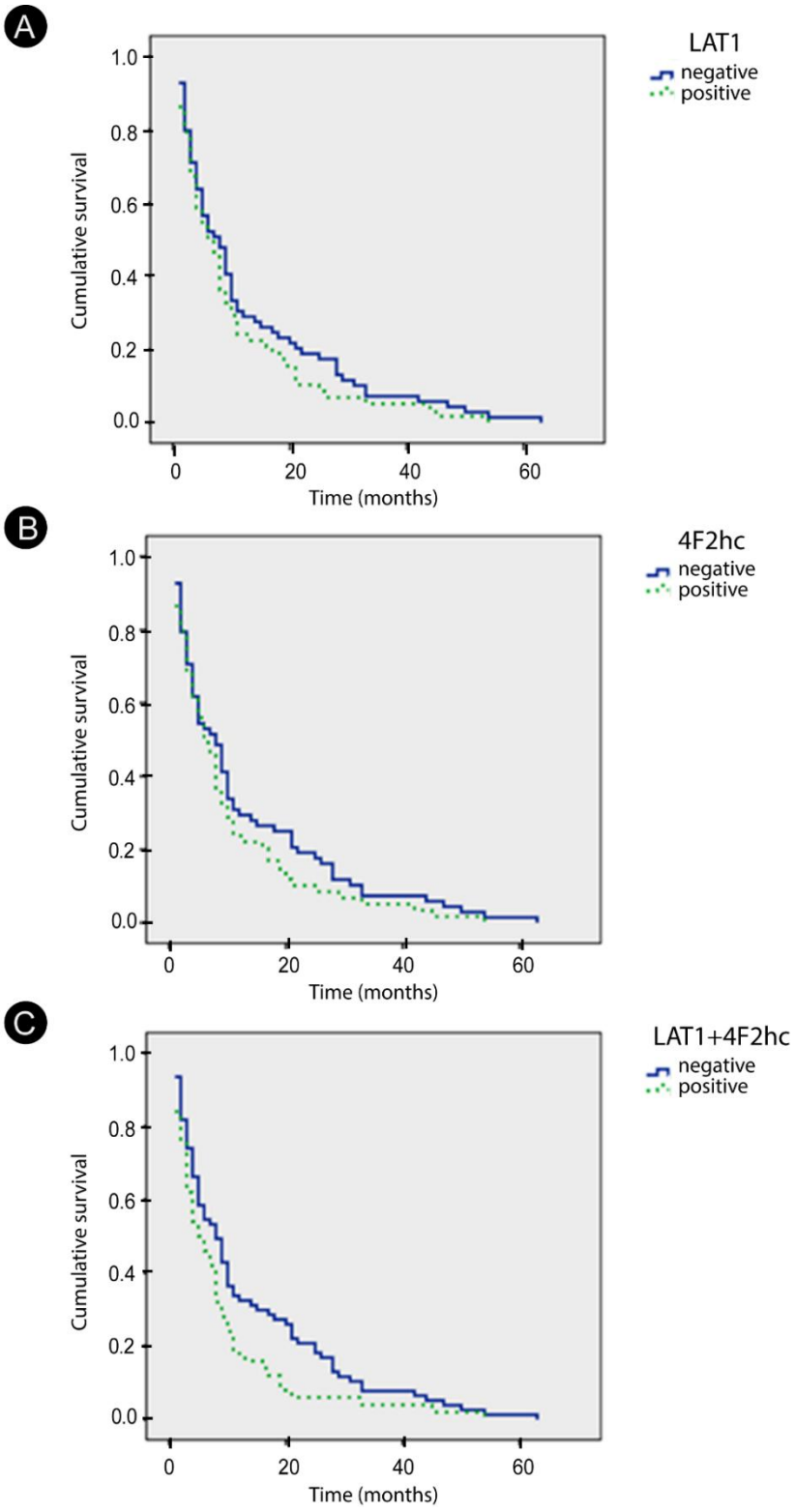

Figure 5. Kaplan-Meier survival analyses of intrahepatic cholangiocarcinoma (ICC) patients with LAT1 (A), 4F2hc (B), and LAT1+4F2hc (C) expressions.

Table 3. Multivariate analysis of prognostic factors influencing the overall survival

Prognostic variables	*p*-value	Hazard ratio	95% CI
TNM stage	**0.040**	2.110	1.035-4.300
T stage	0.833	0.959	0.649-1.417
N stage	0.379	1.225	0.779-1.927
M stage	0.846	0.922	0.409-2.081
LAT1	0.707	0.864	0.404-1.849
4F2hc	0.754	0.890	0.428-1.848
LAT1 + 4F2hc	0.699	1.246	0.408-3.805

Note: Hazard ratio, 95% confidence intervals, and two-side *p* values were obtained from the Cox proportional hazards models.

Objective 2: To investigate the role of LAT1 and its molecular mechanisms for inhibition of tumor progression in both in vitro and in vivo model

Background and Rationale:

Our preliminary experiments found that knockdown of LAT1 in KKU-M213 cells by lentiviral-shRNA suppressed cell proliferation and motility, however the molecular mechanisms are unknown. Interestingly, we also found that knockdown of LAT1 in CCA cells suppressed 4F2hc expression. 4F2hc, a chaperone for proper recruitment of LAT1 to plasma membrane, is highly expressed in various types of cancers and contributes to the formation of tumor through its association partners such as integrin and xCT. Integrin and xCT play an important role in tumor progression and maintaining intracellular glutathione (GSH) levels (a major antioxidant in various malignant tumors), respectively. Therefore, we aim to investigate the molecular mechanisms underlying suppression of cell proliferation and motility mediated by LAT1 knockdown both *in vitro* and *in vivo* models, emphasizing the 4F2hc-mediated signaling pathways.

Part I: To investigate the molecular mechanisms for inhibition of tumor progression using lentiviral shRNA-mediated LAT1 knockdown in CCA cells.

Experiment 2.1: The expressions of 4F2-associating partners in lentiviral shRNA-mediated LAT1 knockdown human CCA cell line by quantitative real time PCR and western blotting

Specific aim: To investigate the effect of LAT1 knockdown on the expressions of 4F2-associating partners, xCT, GLUT1, and integrin $\beta 1$

Method:

Human cholangiocarcinoma (CCA) cell line, KKU-M213 cells were kindly provided by Prof. Dr. Banchop Sripa (Department of Pathology, Faculty of Medicine, Khon Kaen University), respectively. KKU-M213 cells were cultured in Ham's F-12 medium (Invitrogen, CA, USA) supplemented with 10% FBS and maintained at 37 °C in a humidified 95% air and 5% CO_2 incubator. LAT1 knockdown was achieved by transduction with lentivirus, using LentiLox 3.7 system. KKU-M213 cells were seeded at a density of 2.5 x 10^5 cells per 60 mm petri dish and incubated for 24 h. Then, cells were infected by shRNA-LAT1 or shRNA-control lentiviral particles at MOI 1. To validate the knockdown efficiency, total RNAs and protein lysates were extracted after infection for 48 h and the mRNA and protein expressions of LAT1 and 4F2hc were examined by quantitative real time PCR and western blotting, respectively. Stable LAT1 knockdown cells were selected by culturing cells in medium containing antibiotic G418. The effect of LAT1 knockdown on the expressions of the indicated molecules were also examined by quantitative real time PCR or western blotting.

Results and Discussion:

Previous study reported that Thai CCA patient-derived cell line namely KKU-M213 exhibited the highest cell motility and invasion characteristics (Menakongka and Suthiphongchai, 2010). Therefore, KKU-M213 cells were chosen in our study. To study role of LAT1 in KKU-M213 cells, we generated stable and transient LAT1 knockdown by using lentivirus shRNA targeting LAT1 in KKU-M213 cells. By using quantitative real time PCR, the mRNA levels of LAT1 and 4F2hc were significantly decreased approximately 80% (Figure 6A), and 60% (Figure 6B), respectively, in stable LAT1 knockdown KKU-M213 cells (shLAT1-786.12) compared to stable scrambled knockdown cells (shLAT1-S786.1). The total protein lysate from stable LAT1 knockdown KKU-M213 cells was separated on SDS-PAGE and detected with anti-SLC7A5 and anti-4F2hc (anti-CD98) antibodies. Likewise, the LAT1 and 4F2hc protein levels were also significantly decreased approximately 40%

in stable LAT1 knockdown KKU-M213 cells compared to control scramble cells (Figure 2, C-E). In consistent, the expression of 4F2hc was significant reduced after transient knockdown of LAT1 in KKU-M213 cells (Figure 7).

4F2hc is a multifunctional protein which has been reported to interact and regulate several proteins including xCT (a plasma membrane cysteine/glutamate exchanger), glucose transporter 1 (GLUT1), and integrin β1. The function of 4F2hc and their associating protein is identified to be significantly linked to tumor pathogenesis and carcinogenesis. We, therefore, investigated the effect of stable LAT1 knockdown on xCT, GLUT and integrin β1 expression. For xCT, cell lysates were separated on SDS-PAGE in the presence [DTT(+)] and absence [DTT(-)] of DTT followed by staining with anti-xCT antibody. As shown in Figure 8, The ~130 kDa bands corresponding to xCT-4F2hc heterodimer complex were detected in the absence of DTT [DTT(-)]. However, under the reducing condition [DTT(+)], xCT was detected at ~35 kDa bands corresponding to the monomeric form of xCT. This result indicates that xCT forms the heterodimeric complex with 4F2hc via disulfide bonds in KKU-M213 cells. The expression of xCT was only slightly increased in LAT1 stable knockdown KKU-M213 cells when compared to the scrambled negative control cells. Moreover, the expressions of GLUT1 protein and mRNA were not different between LAT1 stable knockdown and scrambled negative control cells (Figure 9). Similar to that of xCT, the expression of integrin β1 was only slight increase in KKU-M213 stable knockdown LAT1 compared to the stable scrambled shRNA (Figure 10).

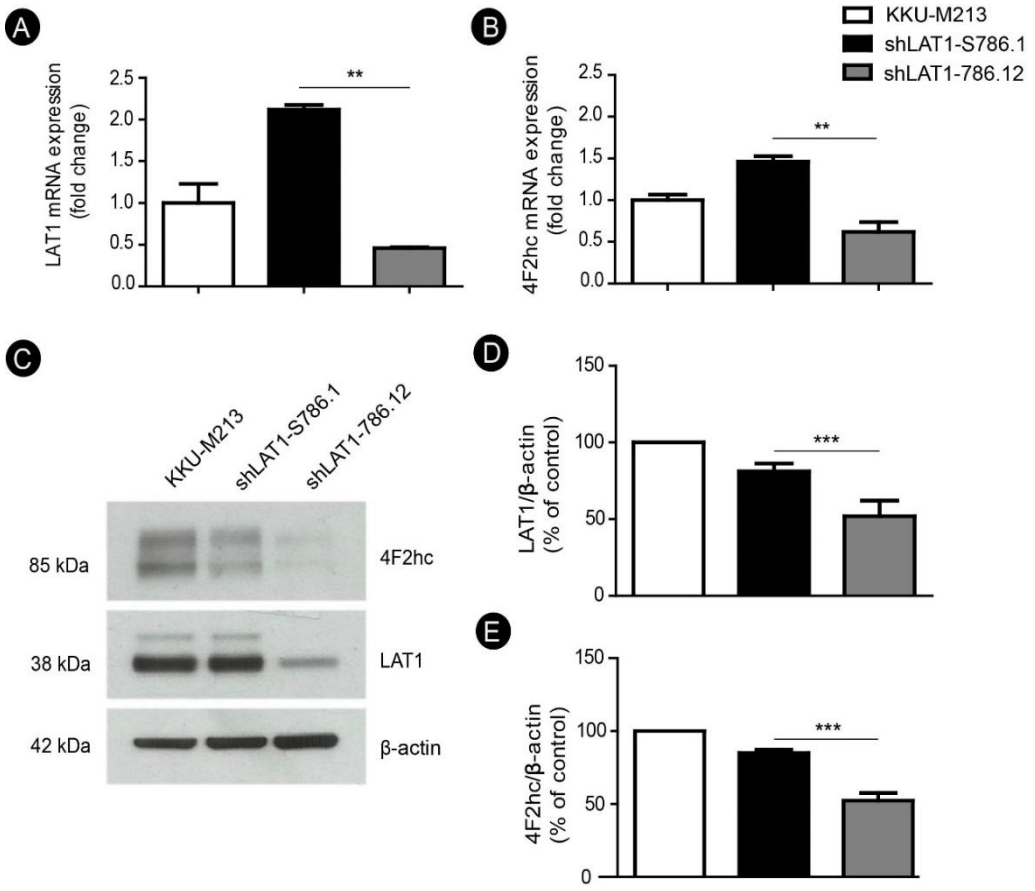

Figure 6. Establishment stable LAT1 knockdown KKU-M213 cells. (A-B) Reduction of LAT1 and 4F2hc mRNA expression in stable LAT1 knockdown KKU-M213 cells. Total RNAs were isolated from KKU-M213 cells stable expression shLAT1-786.12 and shLAT1-s786.1 (scramble). The expressions of LAT1 (A) and 4F2hc (B) were examined by quantitative real time PCR. The results were means ± SEM (n=3) and represented as fold changed compared to negative control (scramble). **, $p < 0.01$ vs negative control (ANOVA). (C-E) Reduction of LAT1 and 4F2hc protein expression in LAT1 stable knockdown KKU-M213 cells. Western blot analysis was performed on total cell lysates using anti-SLC7A5 (LAT1) anti-4F2hc and anti-β-actin antibodies. Bar graphs show the mean normalized densitometry values of LAT1 (D) and 4F2hc (E). Data are means ± SEM (n=3) and presented as % of control. ***, $p < 0.001$ vs negative control (scramble) (ANOVA).

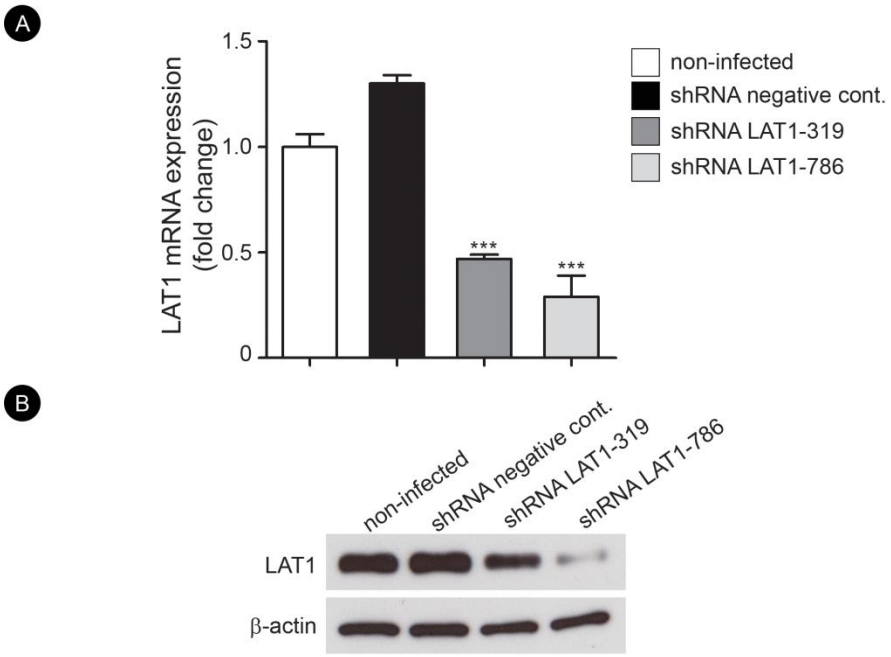

Figure 7. The transient knockdown L-type amino acid transporter 1 (LAT1) in KKU-M213 cells by lentiviral shRNA. (A) Suppression of LAT1 mRNA by shRNA targeted LAT1. By quantitative real time PCR, the expression of LAT1 mRNA was reduced in LAT1 knockdown cells. (B) Suppression of LAT1 protein by shRNA targeting LAT1. Western blot analysis was performed on LAT1 knockdown KKU-M213 cell lysates using anti-SLC7A5 and anti-β-actin antibodies. The results were represented as means ± SEM (n=3). ***, $p < 0.001$ vs shRNA negative control infected cells.

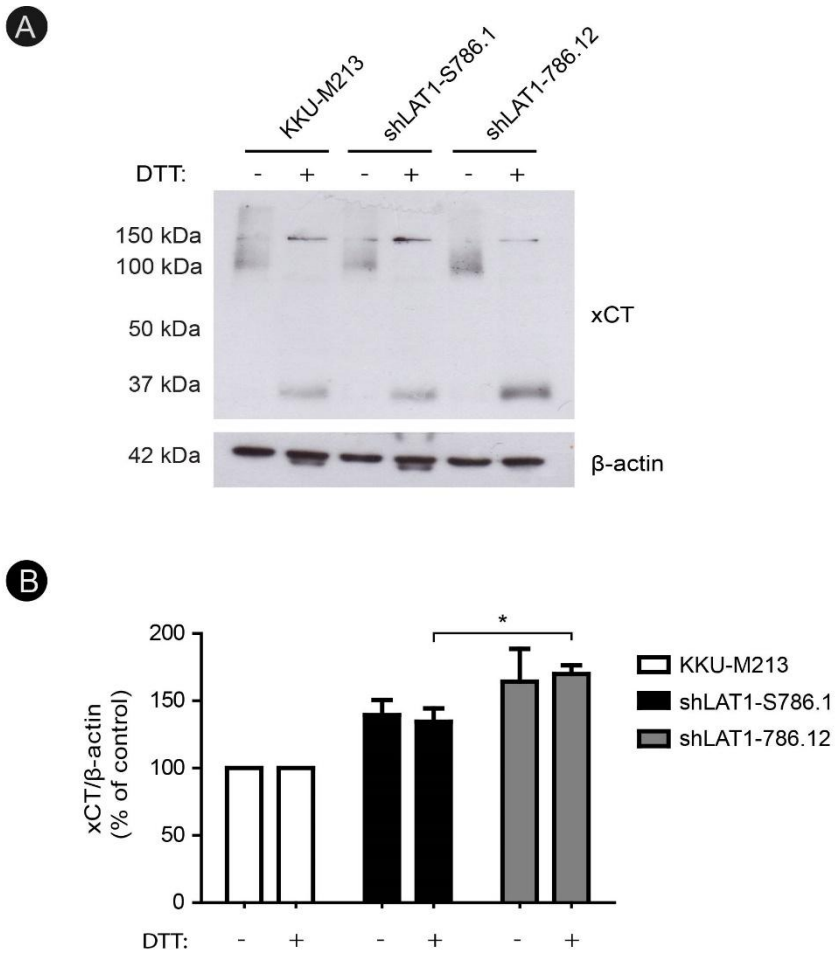

Figure 8. The expression of xCT in stable LAT1 knockdown KKU-M213 cells. (A) Expression of xCT protein in stable LAT1 knockdown KKU-M213 cells. Cells were lysed with modified RIPA lysis buffer and subjected for western blotting in the presence [DTT (+)] or absence [DTT (-)] of DTT with anti-xCT and anti-β-actin antibodies. (B) Bar graphs show the mean normalized densitometry values and the corresponding standard deviations for xCT. The results are represented as means ± SEM (n=3). *, $p < 0.05$ vs scramble shRNA stable cells (ANOVA).

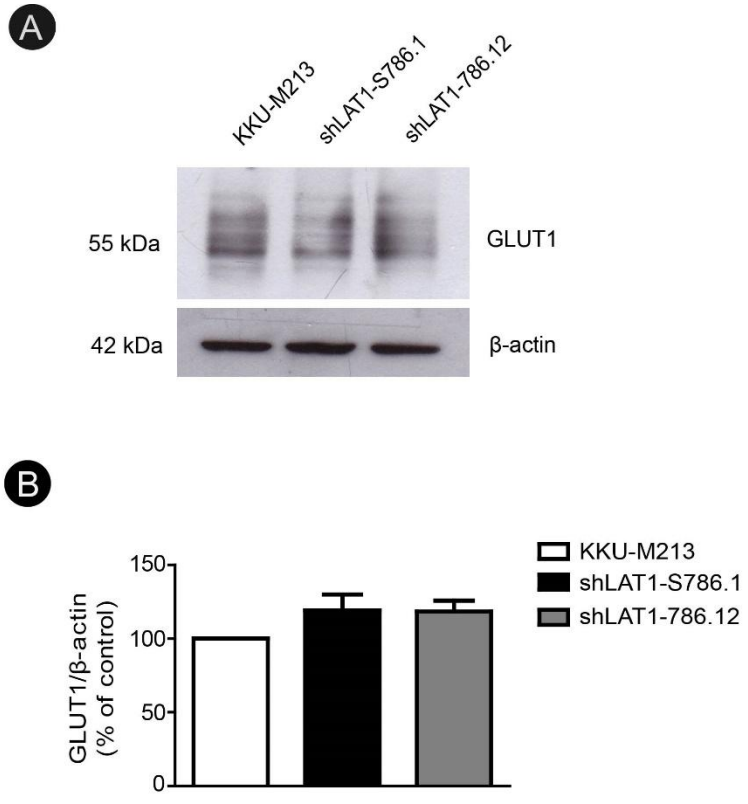

Figure 9. Expression of GLUT1 in stable LAT1 knockdown KKU-M213 cells. (A) Expression of GLUT1 protein in stable LAT1 knockdown KKU-M213 cells. Cells were lysed with modified RIPA lysis buffer and subjected for western blotting with anti-GLUT1 and anti-β-actin antibodies. (B) Bar graphs show the mean normalized densitometry values and the corresponding standard deviations for GLUT1. The results are represented as means ± SEM (n=3) compared to scrambled shRNA stable cells.

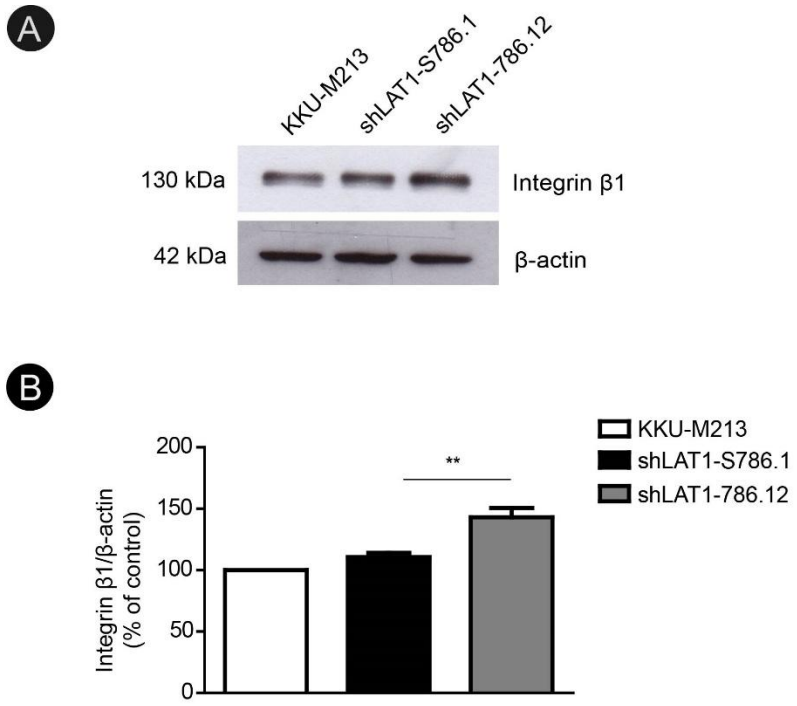

Figure 10. Expression of integrin β1 expression in stable LAT1 knockdown KKU-M213 cells. (A) Expression of integrin β1 protein in stable LAT1 knockdown KKU-M213 cells. Cells were lysed with modified RIPA lysis buffer and subjected for western blotting with anti-integrin β1 and anti-β-actin antibodies. (B) Bar graphs show the mean normalized densitometry values and the corresponding standard deviations for integrin β1. The results are represented as means ± SEM (n=3). **, $p < 0.01$ vs scrambled shRNA stable cells (ANOVA).

Experiment 2.2: Cellular localization of 4F2-associating partners in LAT1 knockdown human CCA cells by confocal microscopy

Background: 4F2hc serves as a chaperone for recruitment of its partners to the plasma membrane. It has been reported that lacking of 4F2hc expression, LAT1 failed to be sorted to plasma membrane and accumulated in intracellular without effecting total protein expression.

Specific aim: To investigate the effect of LAT1 knockdown on cellular localization of 4F2-associating partners

Method:

KKU-M213 cells will be cultured on glass coverslips in 24-well culture plates and infected by shRNA-LAT1 or shRNA-control lentiviral particles. 48 h after infection, cells will be fixed, permeabilized and stained with indicated antibodies overnight before staining with Alexa Fluor tagged secondary antibody. The stained cells will be visualized with confocal laser microscopy (FV10i/w, Olympus).

Results and Discussion:

The effect of stable LAT1 knockdown on LAT1 and 4F2hc expression was further confirmed by confocal immunofluorescence. Consistent with real time PCR and western blotting results, localization of LAT1 and 4F2hc on the plasma membrane was reduced in stable LAT1 knockdown KKU-M213 cells (Figure 11). In addition, the localization of β1-integrin was examined. As shown in Figure 12, in consistent with western blot analysis, the plasma membrane expression of β1-integrin was increased. Although the precise explanation is not known, the up-regulation of β1-integrin might be due to the adaptation of these cells after long-term LAT1 knockdown. In should be noted that, the signals was also observed in nucleus suggesting the non-specific activity of this antibody. We were unable to investigate the localization of xCT in LAT knockdown KKU-M213 cells because the obtained antibody is not recommended for immunofluorescence study. Taken together (experiment 2.1 and 2.2), these results suggest that the regulation of 4F2hc-associating proteins in LAT1 knockdown CCA cells is not simple controlled by the level of 4F2hc. Therefore, the precisely understanding the underlying mechanism is required.

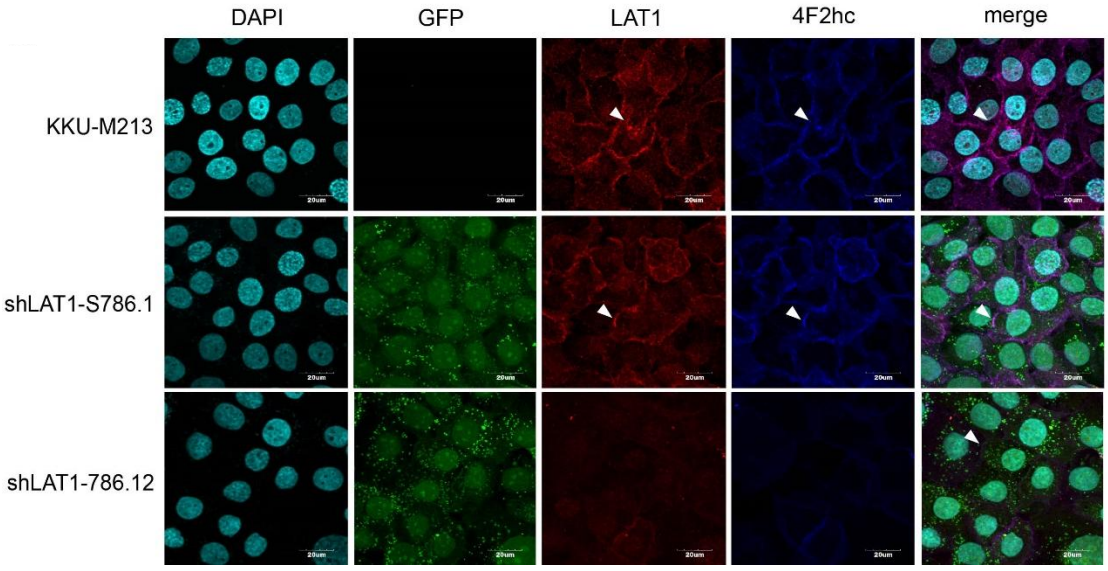

Figure 11. Plasma membrane localization of LAT1 and 4F2hc in KKU-M213 cells stable knockdown LAT1. Cells were fixed and stained with anti-LAT1 (red), anti-4F2hc (blue). DAPI was stained for nuclear marker (light blue). The GFP represents cells infected by lentiviral shRNA. The plasma membrane localization (arrows head) of LAT1 and 4F2hc was reduced in stable LAT1 knockdown cells, bar 20 μm.

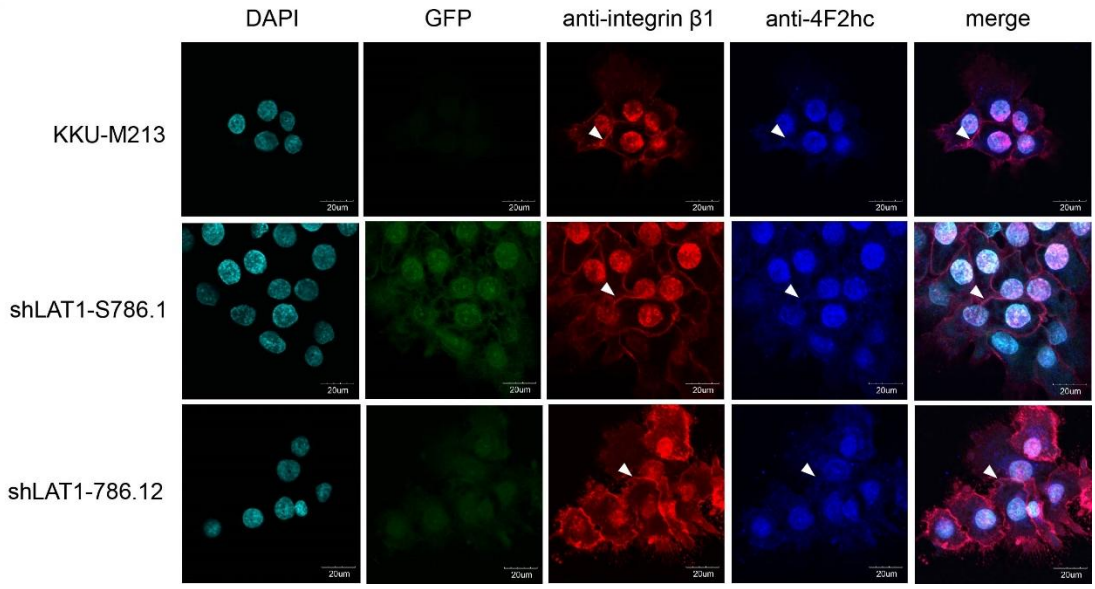

Figure 12. Plasma membrane localization of integrin β1 and 4F2hc in stable LAT1 knockdown KKU-M213 cells. Protein localization was analyzed by confocal microscopy. Stable LAT1 knockdown KKU-M213 and shRNA negative control cells were fixed and stained with anti-integrin β1 (red) and anti-4F2hc (blue) antibodies. DAPI was stained for nuclear marker (light blue). The green color represents the cell infected by lentiviral shRNA. The plasma membrane localization (arrows head) of 4F2hc was reduced in LAT1 knockdown cells whereas integrin β1 was not, bar 20 μm.

Experiment 2.3: The effect of LAT1 knockdown on transport function of LAT1/4F2hc and xCT/4F2hc in human CCA cell lines by uptake assay

Background and specific aim: 4F2hc is important for functional expression of LAT1 and xCT amino acid transporters. Therefore, we would like to investigate the effect of 4F2hc downregulation in LAT1 knockdown cells on transport activity of xCT.

Method:

Stable LAT1 knockdown KKU-M213 cells were cultured 48-well culture plates for 48 h. Cells were washed twice with D-PBS and then incubated with D-PBS containing 1 μM [^{14}C]L-leucine (substrate for LAT1-4F2hc) for 2 min and 100 μM [^{14}C]L-cystine (substrate for xCT-4F2hc) for 3 min. Uptake rate was stopped by washing with ice-cold D-PBS 3 times. Cells were lysed and counted by scintillation spectrometry.

Results and Discussion:

LAT1 preferentially transports large neutral amino acids especially leucine, the essential amino acids which is necessary for cancer cell growth. LAT1 belongs to a system L amino acid transporter. BCH, system L inhibitor has been reported to reduce leucine uptake-mediated by LAT1 and suppressed growth of several cancer cells. Therefore, we next investigated the effect of LAT1 knockdown on leucine transport activity. As shown in figure 13A, [^{14}C]L-leucine (1μM) uptake was significant decreased (approximately 20%). Moreover, in the present of 100 μM BCH, the [^{14}C]L-leucine uptake was further reduced suggesting the transport was mediated by system L. Although the expression of LAT1 protein was suppressed by 80% (Figure 6), however the LAT1-mediated leucine uptake is reduced only 20%. This observation might be due to the upregulation of other isoforms of system L amino acid transporters. We next investigated the compensatory response of stable LAT1 knockdown on the expression of other isoforms of system L amino acid transporters. The mRNA expressions of LAT2, LAT3, and LAT4 were examined by quantitative real time PCR. We found that there the expressions of other isoforms of system L amino acid transporters were not affected in stable LAT1 knockdown KKU-M213 cells (Figure 13B). The similar results were obtained by transient knockdown LAT1 (data not show). These results indicate no compensatory response on the expression of other isoforms of system L amino acid transporters after LAT1 knockdown in KKU-M213 cells. In consistent with Figure 4, knockdown of LAT1 has no effect on cyctine uptake (Figure 14). Collectively, these results indicate that stable knockdown LAT1 suppressed leucine uptake without any effect on the expression of another isoforms of system L amino acid transporters and the transport activity of xCT, the 4F2hc associating protein.

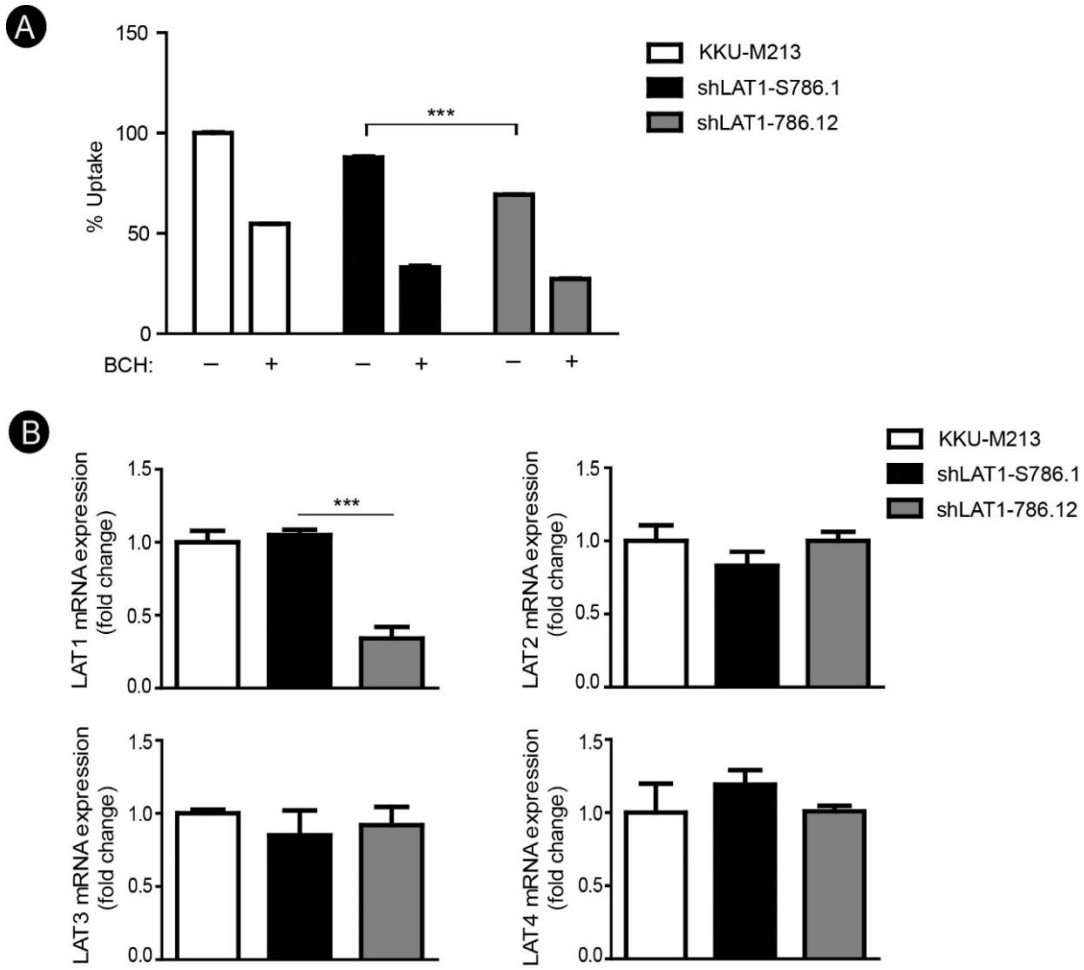

Figure 13. The effect of stable LAT1 knockdown on leucine transport in KKU-M213 cells. (A) Leucine uptake in LAT1 stable knockdown KKU-M213 cells. [^{14}C]L-Leucine (1 μM) uptake was performed in stable LAT1 knockdown KKU-M213 cells in the presence or absence of 100 μM BCH for 2 min. The results are means ± SEM (n = 3) and presented as % of uptake compared to negative control (scramble). ***, $p < 0.001$ vs negative control (ANOVA). (B) The mRNA expressions of system L amino acid transporters in stable LAT1 knockdown KKU-M213 cells. Total RNAs were isolated from KKU-M213 cells stable expression shLAT1-786.12 and shLAT1-s786.1 (scramble). The expressions of LAT1, LAT2, LAT3, and LAT4 were examined by quantitative real time PCR. The results are means ± SEM (n=3) and represented as fold change compared to negative control (scramble). ***, $p < 0.001$ vs negative control (ANOVA).

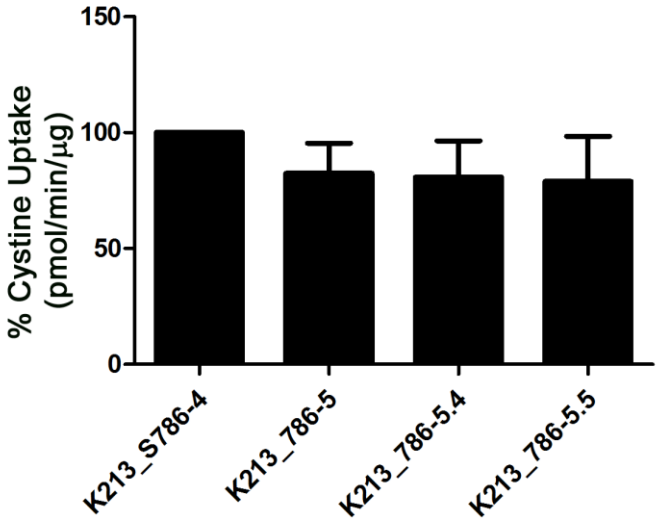

Figure 14. The effect of stable LAT1 knockdown on cystine transport in KKU-M213 cells. [^{14}C]L-Leucine (100 µM) uptake was performed in several clones of stable LAT1 knockdown KKU-M213 cells for 3 min. The results are means ± SEM (n = 3) and presented as % of uptake compared to negative control (scramble).

Experiment 2.4: To study the effect of LAT1 knockdown on malignant phenotypes and its-related signaling pathways in human CCA cell line

Background and specific aim:

Previous studies reported that 4F2hc promoted tumorigenesis by activation of integrin signaling pathway. In addition, leucine uptake mediated by LAT1 is required for cancer cell growth through mTOR activation. We, therefore, aim to examine whether LAT1 knockdown CCA cells suppresses integrin and mTOR signaling pathways.

Method:

The effect of stable or transient LAT1 knockdown on malignant phenotypes (proliferation, survival, migration and invasion) was investigated in KKU-M213 cells by BrdU, clonogenic, migration and invasion assays, respectively. For their underlying mechanisms, stable or transient LAT1 knockdown KKU-M213 cells were cultured with FBS-free medium for 16 h and analyzed by western blotting with anti-p-p70S6K (Thr389), anti-p70S6K, anti-p-Akt (Ser473), anti-Akt, anti-p-ERK1/2 (Thr202/Tyr204), and anti-ERK1/2 antibodies, the proteins in mTOR and integrin signaling pathways.

Results and Discussion:

We next determined the effect of stable LAT1 knockdown on the proliferative malignant phenotype of CCA cells by MTT and BrdU incorporation assays. We found that stable knockdown LAT1 was not effect on CCA cell proliferation (Figure 15A). The effect of stable LAT1 knockdown on CCA cell proliferation was further confirmed by BrdU incorporation assay. The result was consistent with MTT assay, stable LAT1 knockdown in CCA cells had no effect on BrdU incorporation (Figure 15B). These results indicate that stable knockdown of LAT1 has no effect on CCA cell proliferation. This result was consistent with our unpublished data that inhibition of leucine transport by BCH in KKU-M213 cells exhibited only slight decreased in cell proliferation. Whereas, BCH treatment markedly suppressed HuCCA-1 and KKU-100 cell proliferation, suggesting the specific roles of LAT1 in KKU-M213 cells. Moreover, we also investigated the colony formation ability of stable LAT1 knockdown KKU-M213 cells by using clonogenic assay which is a technique based on the ability of single cancer cell to develop a surviving colony that widely used to evaluation the oncogenic potential of cancer cells. The result revealed that stable LAT1 knockdown cells formed lower number of colony (approximately 40%) than that of stable scrambled shRNA cells (Figure 16, A-B). This result suggests that long-term knockdown LAT1 affects the ability of CCA cells to survive rather than cell proliferation property. However, the underlying mechanism is unclear.

Stable knockdown LAT1 has no effect on cell proliferation might be due to adaptive response in this cell line. We therefore investigate the effect of transient LAT1 knockdown by lentiviral shRNA on CCA cell proliferation by MTT assay. Cells were infected by lentiviral shRNA for 48 h and analyzed for number of viable cells by MTT assay every 2 day up to 8 days. As shown in Figure 17A, suppression of LAT1 by lentiviral shRNA was not significantly effect on cell proliferation when compared to lentiviral shRNA negative control infected cells. Moreover, we further confirmed the effect of lentiviral shRNA after infection for 8 days on the suppression of LAT1 expression. By quantitative real time PCR, we found that LAT1 expression was reduced by 50% at day 8. We, next investigated the effect of transient LAT1 knockdown on KKU-M213 cell motility. For cell migration, the infected cell was plated into the upper chamber of trans-well cultured with complete medium containing 10% FBS. After cultured for 12 h, the number of migrated cells was determined. As shown in Figure 18A, number of cell migration was significantly decreased in two shRNA LAT1-319 and -786 infected cells approximately 30% and 40%, respectively, compared to shRNA negative control infected cells. For cell invasion, the infected cell was plated into the upper chamber of trans-well

coated with matrigel and cultured with complete medium containing 10% FBS for 12 h. As shown in Figure 18B, cell invasion was significantly decreased in shRNA LAT1-319 and -786 infected cells (approximately 40%) compared to shRNA negative control infected cells. These results indicate that suppression of LAT1 by lentiviral shRNA results in reduction of KKU-M213 cell survival and motility but has no effect on cancer cell proliferation. It should be noted that roles of LAT1 may be cell type-dependent. Therefore, roles of LAT1 and 4F2hc should be investigated in other type of CCA cell lines.

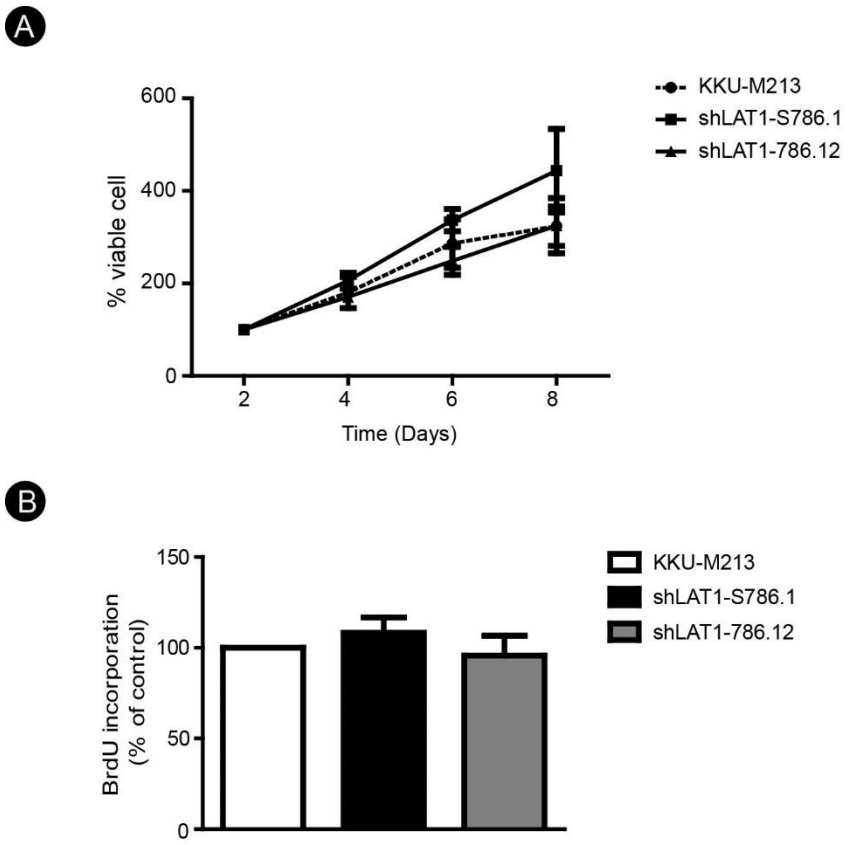

Figure 15. The effect of stable LAT1 knockdown on KKU-M213 cell proliferation. (A) The effect of stable LAT1 knockdown on KKU-M213 cell viability by MTT assay. Stable LAT1 knockdown and negative control KKU-M213 cells were plated on 96-well plates. After incubation, cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) every 2 day for 8 days. (B) The effect of stable LAT1 knockdown on KKU-M213 cell proliferation by BrdU incorporative assay. Stable LAT1 knockdown and negative control KKU-M213 cells were plated on 96-well plates. After incubation for 6 days, cell proliferation was determined by BrdU assay. The results are means ± SEM (n=3) and represented as % compared to scramble shRNA negative control cells.

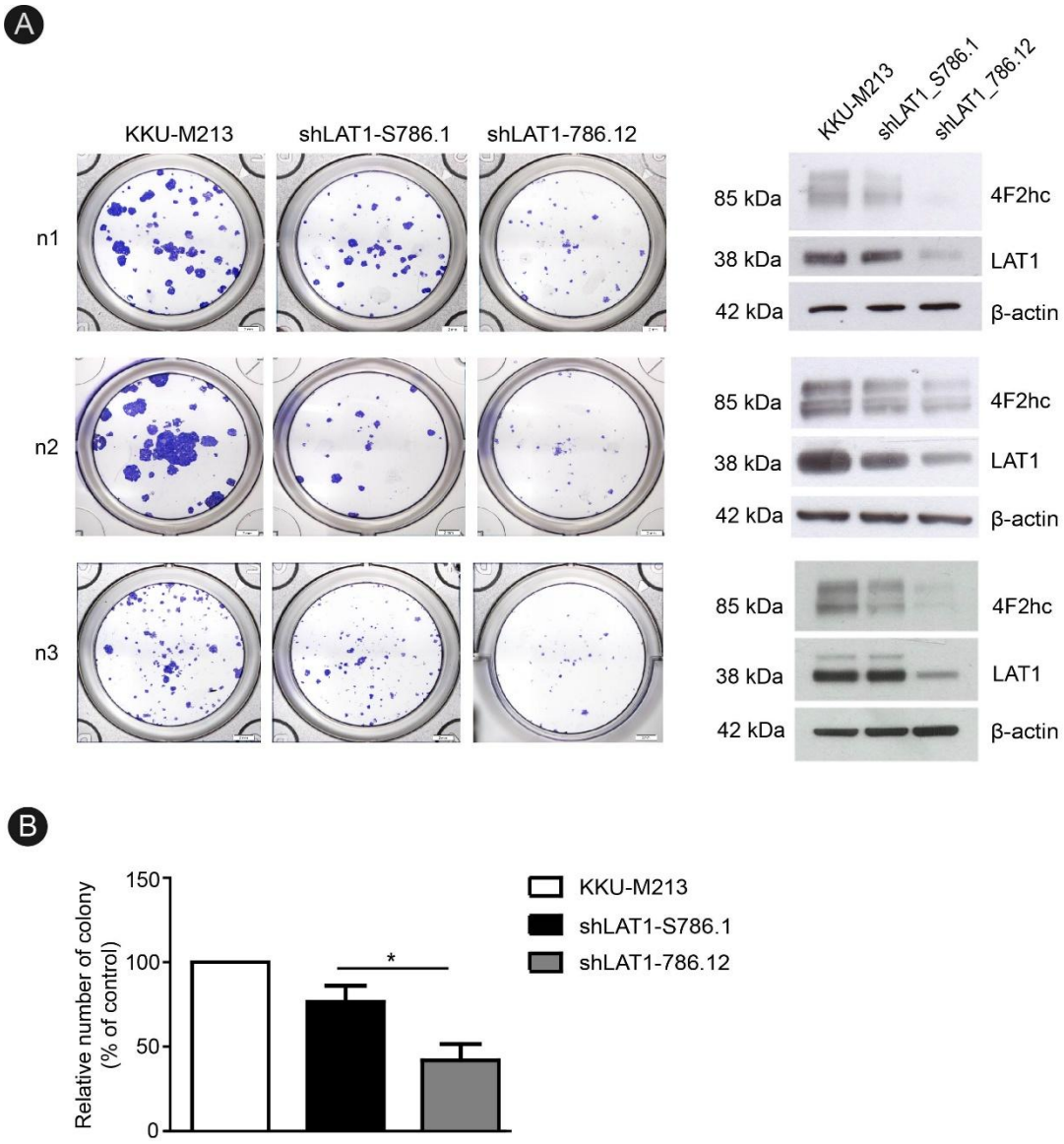

Figure 16. The proliferative indefinitely properties of stable knockdown LAT1 in KKU-M213 cells. (A) Stable knockdown LAT1 suppresses the number of colony formation in clonogenic assay. Stable knockdown LAT1, scrambled shRNA and non-infected KKU-M213 cells were plated on 24-well plate (100 cells/well) and cultured for 2 weeks. Colony-formed cells were stained with 0.5% crystal violet and visualized by Macro Zoom Imaging System (MVX10) (Olympus, Japan). The expression of LAT1 and 4F2hc of the corresponding cells was shown in the right. (B) The number of colony is quantified and represented as means ± SEM (n = 3). *, $p < 0.05$ vs scrambled shRNA control (ANOVA).

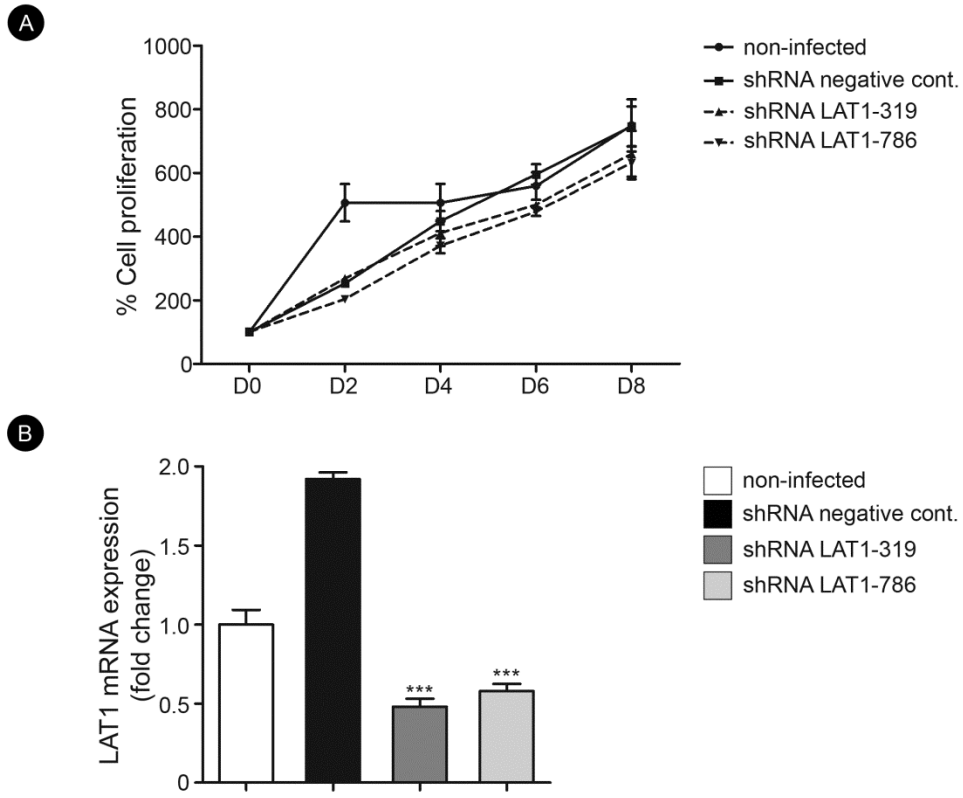

Figure 17. The effect of transient LAT1 knockdown on KKU-M213 cell proliferation. (A) LAT1 knockdown KKU-M213 cells were plated on 96-well plates. After incubation, cell survival was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) every 2 day for 8 days. (B) The effect of LAT1 knockdown on LAT1 expression. The RNA of LAT1 knockdown KKU-M213 cells were isolated after infection for 8 days. The mRNA expression of LAT1 was examined by quantitative real time PCR. The results are represented as means ± SEM (n=3). ***, $p < 0.001$ vs shRNA negative control infected cells.

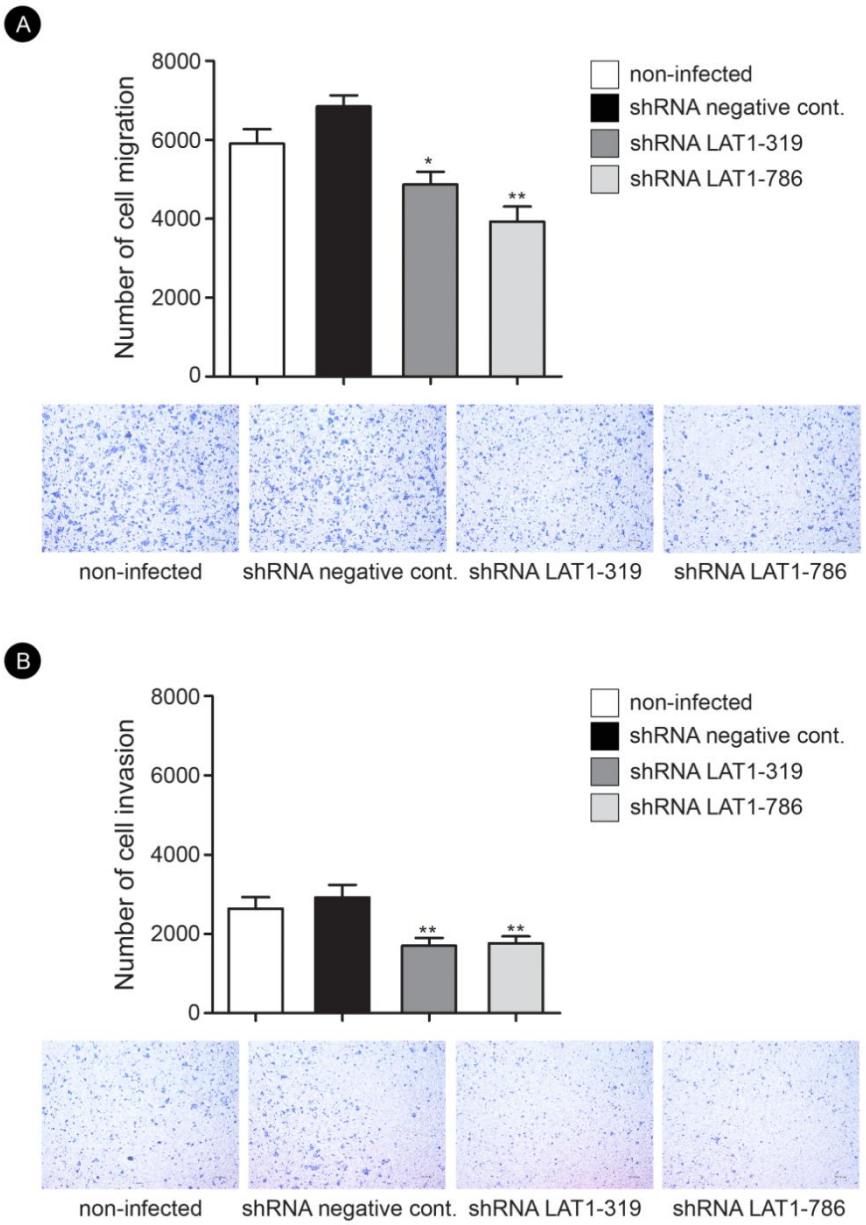

Figure 18. The effects of transient LAT1 knockdown on KKU-M213 cell motility. For invasion and migration assays, cells were plated in the upper chamber of trans-well coated with or without matrigel for invasion and migration assay, respectively. The lower chamber was filled with complete medium containing 10% FBS. After 12 h incubation, numbers of invading (A) and migrating (B) cells were counted. The results are represented as means ± SEM (n=3). *, $p < 0.01$; **, $p < 0.001$ vs shRNA negative control infected cells.

Next, we asked whether the effect of stable LAT1 knockdown on suppression of clonogenic malignant phenotype in human cholangiocarcinoma cells is either through the inhibition of mTOR and integrin signaling pathways. Therefore, we investigated the effect of stable LAT1 silencing on p70S6K activity, an important downstream target of mTOR and phosphorylation of Akt and Erk1/2. We found that long-term suppression of LAT1 had no effect on the phosphorylation of p70S6K at threonine 389 (Thr389) (Figure 19). Whereas, the phosphorylation of Akt at serine473 (Ser473) was significantly decreased in stable LAT1 knockdown cells compared to scrambled shRNA cells (Figure 20). The activity of Erk1/2 signaling pathway was also reduced as demonstrated by decreasing phosphorylation of ERK1/2 at threonine 202 and tyrosine 204 residues (Thr202/Tyr204) (Figure 21). Taken together our results indicate that long-term suppression of LAT1 reduced Akt and Erk1/2 activities that leads to suppress the proliferative indefinitely property of human cholangiocarcinoma cells. We next confirmed the effect of stable LAT1 knockdown by transient knockdown experiment. Consistent results were observed as the stable LAT1 knockdown experiments, the activities of ERK1/2 and p70S6K were significantly reduced as illustrated by the reduction of the levels of ERK1/2 and p70S6K phosphorylation in KKU-M213 cells transient knockdown LAT1 (Figure 12). It should be noted that the suppression of mTOR signaling as demonstrated by reduction of p70S6K phosphorylation was observed only in transient LAT1 knockdown. This discrepancy might be due to the difference in the effectiveness of knockdown between two experiments. These results indicate that suppression of the malignant phenotypes-mediated by LAT1 knockdown may partly be through the inhibition of Akt, ERK1/2 and p70S6K activities.

The previous study showed that microRNA-7 is a 4F2hc negative regulator (Nguyen et al., 2010). Therefore, we investigated the effect of LAT1 knockdown on the expression of microRNA-7 in KKU-M213 cells. As shown in Figure 23, infection KKU-M13 cells with lentiviral shRNA targeting LAT1 for 48 h significant suppressed LAT1 and 4F2hc expression compared to shRNA negative control. Most interestingly, the expression of microRNA-7 was significantly increased in transient LAT1 knockdown KKU-M213 cells compared to the negative control. Our unpublished data revealed that the expression of microRNA-7 did not affect by BCH treatment, suggesting the molecular interaction between LAT1 and microRNA-7. However, how the expression of microRNA-7 is regulated by LAT1 is not known. Collectively, our results indicate that the suppression of CCA cell invasion and migration may be due to the inhibition of 4F2hc by microRNA-7 in LAT1 knockdown cells. It should be noted that not only regulates 4F2hc expression, microRNA-7 regualates several other signaling molecules in cells. Therefore, the suppression of KKU-M213 phenotypes-mediated by LAT1

knockdown might be also through the signaling relation to microRNA-7 upregulation which is 4F2hc-independent pathway.

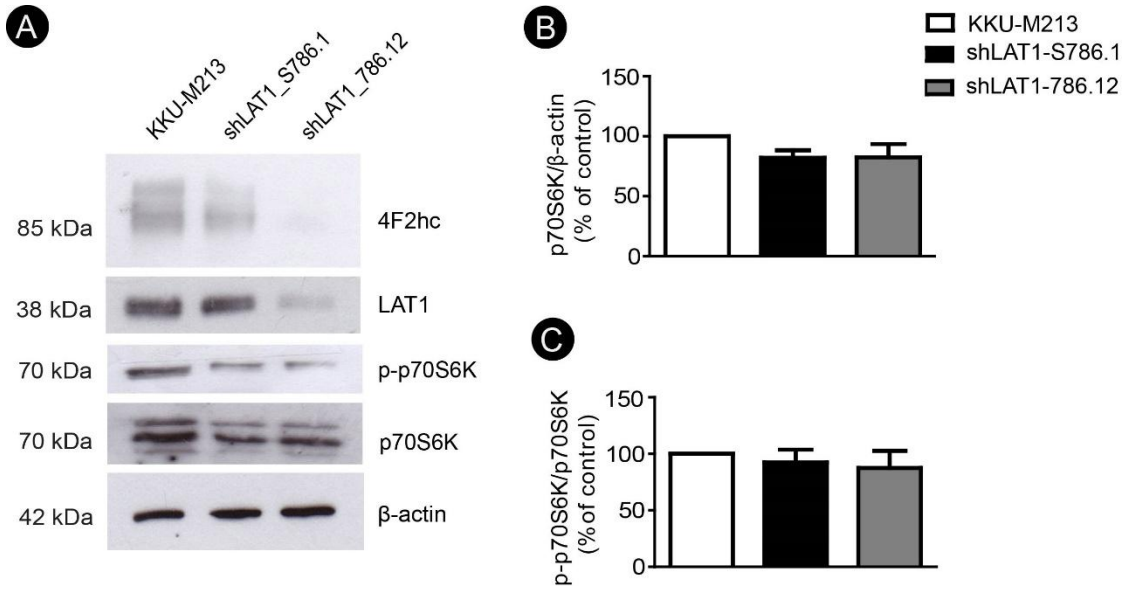

Figure 19. Stable knockdown LAT1 has no effect on mTOR activity. (A) The effect of LAT1 knockdown on mTOR activity. Western blot analysis was performed on LAT1 knockdown and shRNA negative control cell lysates using anti-LAT1, anti-4F2hc, anti-p-p70S6K (Thr389), anti-p70S6K and anti-β-actin antibodies. Bar graphs show the mean normalized densitometry values and the corresponding standard deviations for p70S6K (B) and p-p70S6K (C). Data are means ± SEM (n = 3) and presented as % of control.

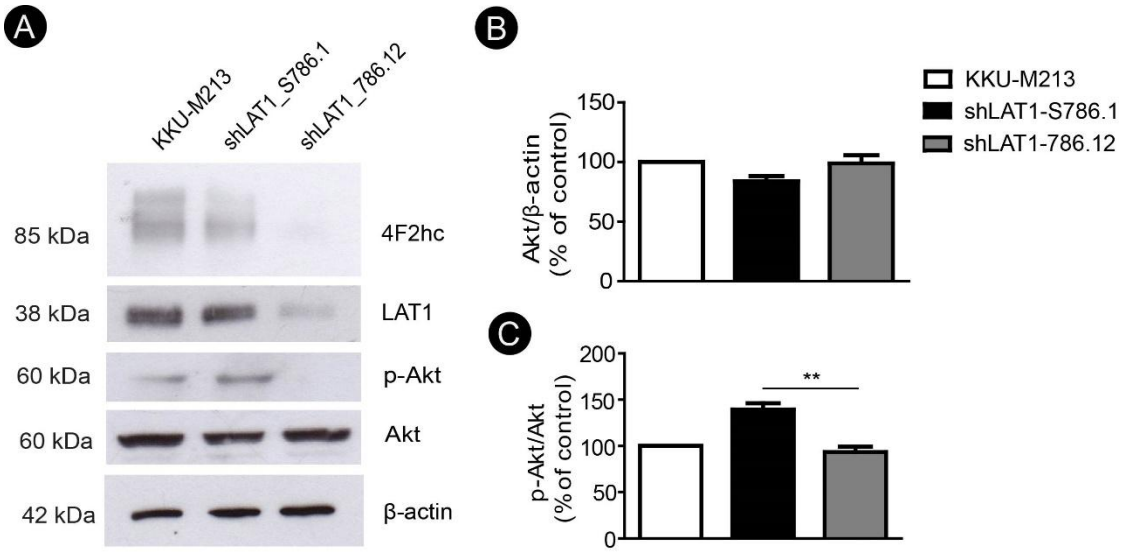

Figure 20. Stable knockdown LAT1 reduces Akt activity. (A) The effect of LAT1 knockdown on Akt phosphorylation. Western blot analysis was performed on LAT1 knockdown and shRNA negative control cell lysates using anti-4F2hc, anti-LAT1, anti-p-Akt (Ser 473), anti-Akt and anti-β-actin antibodies. Bar graphs show the mean normalized densitometry values and the corresponding standard deviations for Akt (B) p-Akt (C). Data are means ± SEM (n = 3) and presented as % of control (ANOVA); **, $p < 0.01$ vs shRNA negative control.

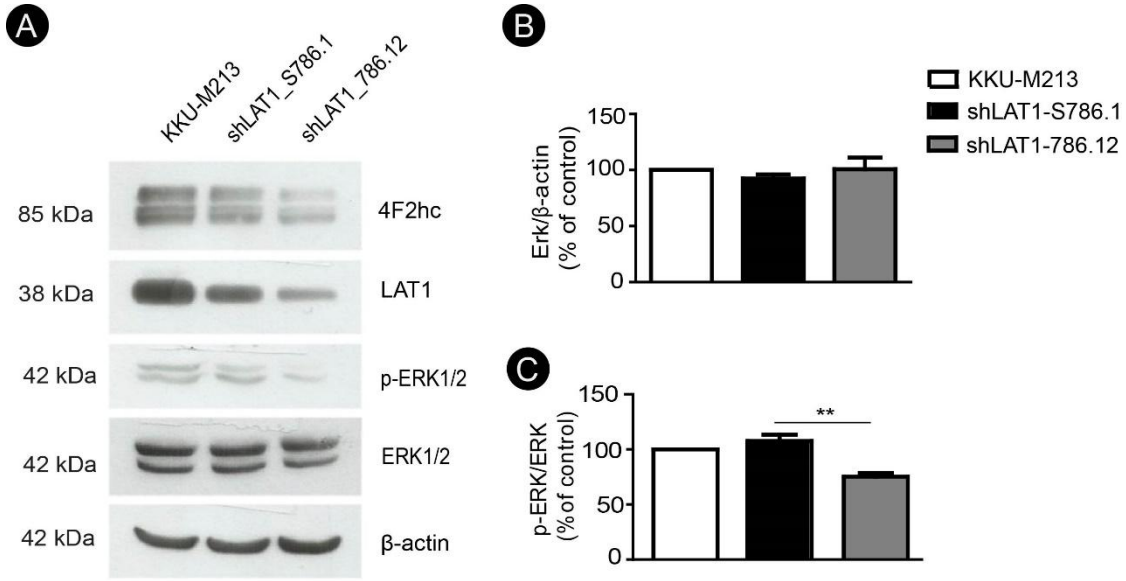

Figure 21. Stable knockdown LAT1 suppresses ERK 1/2 activity. (A) The effect of LAT1 knockdown on Erk1/2 activity. Western blot analysis was performed on LAT1 knockdown and shRNA negative control cell lysates using anti-LAT1, anti-4F2hc, anti-p-Erk1/2 (Thr202/Tyr204), anti-Erk1/2 and anti-β-actin antibodies. Bar graphs show the mean normalized densitometry values and the corresponding standard deviations for ERK1/2 (B), and p-Erk1/2 (C). Data are means ± SEM (n = 3) and presented as % of control (ANOVA); **, $p < 0.01$ vs shRNA negative control

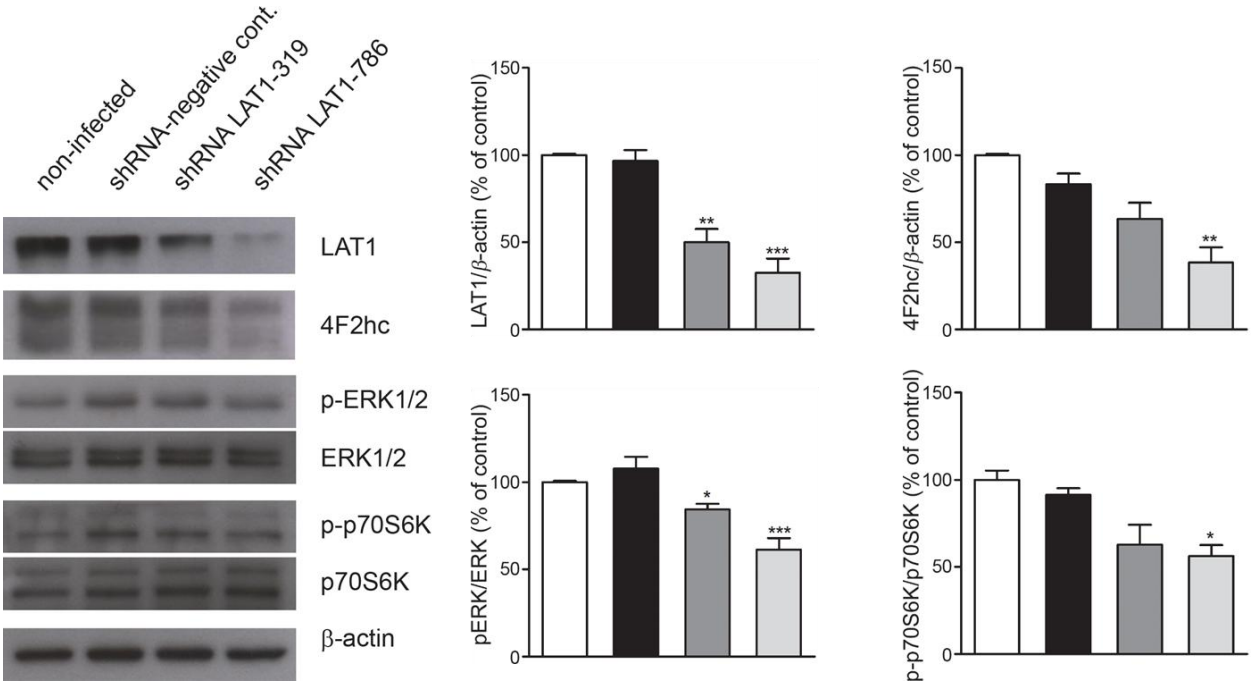

Figure 22. Transient LAT1 knockdown suppresses ERK1/2 and p70S6K activities. Total cell lysates were extracted from KKU-M213 cells after transiently knockdown LAT1 for 48 h and analyzed by western blotting using the indicated antibodies. Bar graphs show the mean normalized densitometry values and the corresponding standard deviations for LAT1, 4F2hc, ERK1/2, and p70S6K. Data are means ± S.E.M. (n=3) and presented as % of control (ANOVA); *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ compared with shRNA negative control infected cells.

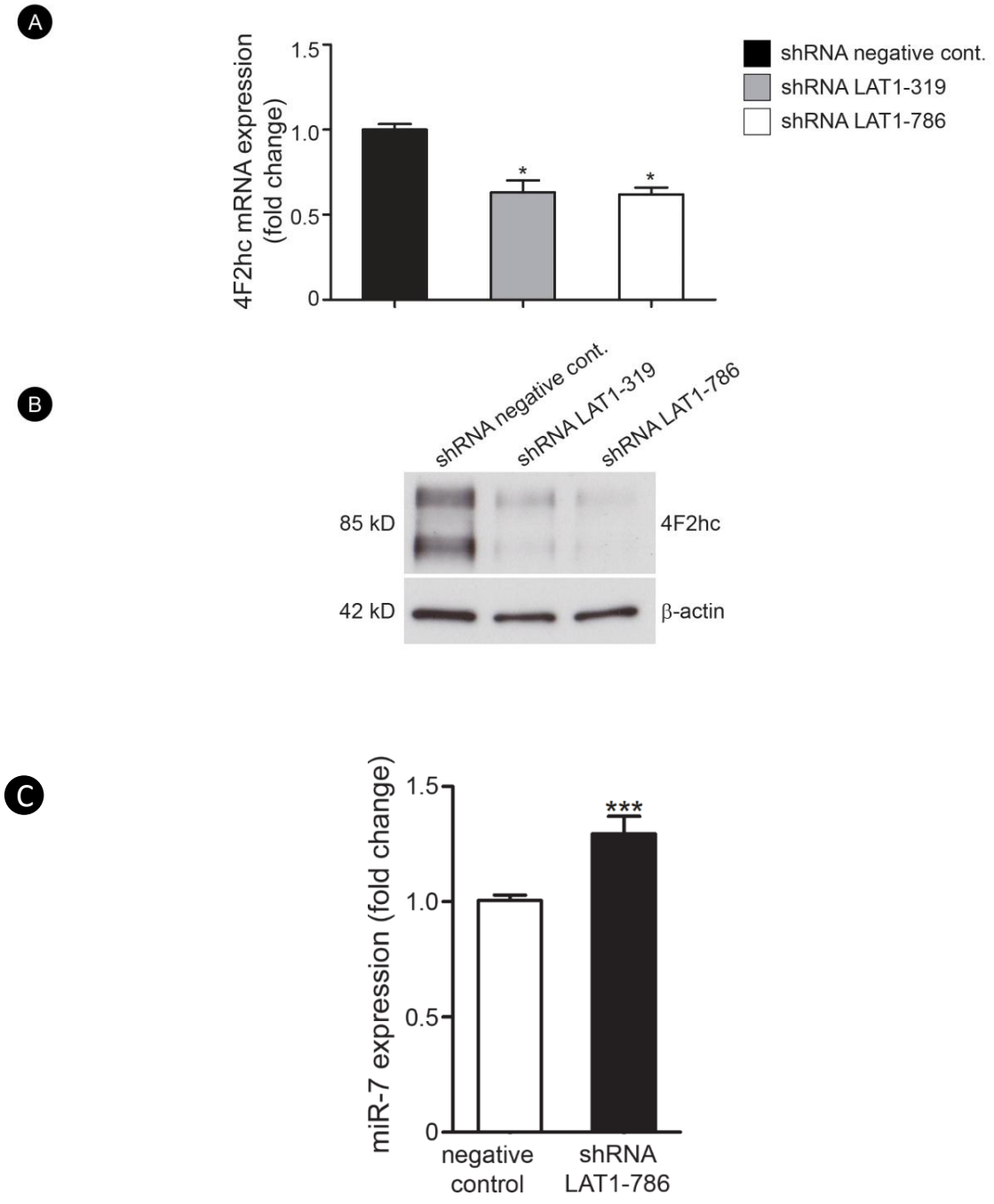

Figure 23. Expression of 4F2hc in transient LAT1 knockdown KKU-M213 cells. (A) Suppression of 4F2hc mRNA expression in LAT1 knockdown CCA cells. By quantitative real time PCR, the expression of 4F2hc mRNA was reduced in LAT1 knockdown KKU-M213 cells. (B) Suppression of

4F2hc protein expression in LAT1 knockdown CCA cells. Western blot analysis was performed on LAT1 knockdown cell lysates using anti-4F2hc and anti-β-actin antibodies. The results are means ± SEM (n=3) and represented as fold change. *, $p < 0.01$ vs shRNA negative control infected cells. (C) Upregulation of mcroiRNA-7 in LAT1 knockdown KKU-M213 cells. The expression of microRNA-7 was measured by quantitative real time PCR normalized by the expression of RNU6-2 gene. The result is means ± S.E.M. (n=3) and presented as fold change (Student's *t* test); ***, $p<0.001$ compared with shRNA negative control infected cells.

Conclusion:

In summary, we found that co-expression of LAT1 together with 4F2hc was significantly correlated with various clinicopathological factors and shorter survival time of ICC patients compared to either the expression of LAT1 or 4F2hc alone. However, from multivariate analysis, only TNM stage exhibited independent prognostic value, thus, to use as prognostic markers for ICC patients, the expression of LAT1 and 4F2hc together with other clinicopathological parameters is suggested. Our study also demonstrated that knockdown of LAT1 suppressed KKU-M213 cell survival and motility. The effects of LAT1 on CCA cells seemed to be cell type specific. To our knowledge, we showed here for the first time that microRNA-7 expression was regulated by LAT1. Upregulation of microRNA-7 observed in LAT1 knockdown cells results in down-regulation of 4F2hc expression. However, the expression of 4F2hc-associating protein was not altered. Therefore, LAT1 knockdown affected KKU-M213 cell invasion and migration might be partly through the inhibition of 4F2hc-ERK1/2-p70S6K signaling pathway. Since microRNA-7 and 4F2hc plays significant roles in normal cell function, systemic targeting microRNA-7 and 4F2hc may harm normal cells. Therefore, specific targeting microRNA-7 and 4F2hc through inhibition LAT1 expression, which is highly expressed in cancer cells, would specifically target cancer cell growth and metastasis without serious adverse effects to normal cells. However, our study highlights several challenging questions. It is not known about specific roles of LAT1 in different type of CCA cells. How expression of microRNA-7 is regulated by LAT1. Recently, we found the expression of 4F2hc in exosomes secreted from KKU-M213 but not from normal cholangiocyte cells. In field of liquid biopsy, the contents in exosome are widely used as cancer biomarkers. Although, it is not known about role of 4F2hc in cancer derived exosomes however, it has the potential to be used as biomarker for cancer patient. Understanding all of these challenging questions would be benefit for CCA patients in the future.

Remarks:

1. xCT-4F2hc heterodimeric amino acid transporter is essential for uptake of cystine required for intracellular GSH synthesis that is important for maintaining the intracellular redox balance in cells. Downregulation of 4F2hc is expected to impair xCT function that may results in GSH depletion and ROS accumulation. However, our experiment found that LAT1 knockdown reduced 4F2hc expression but has no effect on xCT expression and function. Therefore, the effect of LAT1 knockdown on GSH and ROS levels in human CCA cell line is not investigated. In addition, the results of all of knockdown experiments were compared with non lentivirus infected and lentiviral-shRNA control cells to exclude non-specific effects. Hence, the effect of lentivirus infection in normal cells is omitted.
2. The objective in part II is to investigate the effect of LAT1 on CCA cell growth and proliferation in xenograft mouse model. From our results we found that CCA cell growth and proliferation were not affected by both stable and transient LAT1 knockdown. Therefore, the effects of LAT1 knockdown on growth and proliferation of KKU-M213 cells in subcutaneous tumor model is not carried on in this project.

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Downregulation of LAT1 expression suppresses cholangiocarcinoma cell invasion and migration

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ABSTRACT

Currently, there is no effective treatment for cholangiocarcinoma (CCA), which is the most prevalent in the northeastern part of Thailand. A new molecular target for the treatment of CCA is, therefore, urgently needed. Although L-type amino acid transporter 1 (LAT1) is highly expressed in CCA cells, its role in malignant phenotypes of CCA cells remains unclear. This study aimed to investigate the impact of LAT1 on proliferation, migration, and invasion of KKU-M213 cells, the CCA cells derived from Thai patients with intrahepatic cholangiocarcinoma. Results showed that KKU-M213 cells expressed all LAT isoforms (LAT1, LAT2, LAT3 and LAT4). The expressions of LAT1 and its associated protein 4F2hc were highest whereas those of LAT2 and LAT4 were extremely low. Treatment with 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) reduced L-leucine uptake concomitant with an inhibition of cell motility and, to a lesser extent, on cell proliferation. It also induced a time dependent up-regulation of LAT1 and 4F2hc expressions. Similarly, cell migration and invasion, but not proliferation, were reduced in LAT1 knockdown KKU-M213 cells. In addition, silencing of LAT1 inhibited the expressions of 4F2hc mRNA and protein whereas the expression of microRNA-7, the 4F2hc down-regulator, was increased. Furthermore, the phosphorylation levels of ERK1/2 and p70S6K were reduced after LAT1 knockdown. Collectively, these results suggest that suppression of cell invasion and migration in LAT1 knockdown KKU-M213 cells may be partly mediated through the inhibition of the 4F2hc-signaling pathway by the up-regulation of microRNA-7. Based on this finding, LAT1 may be a potential therapeutic target for treating CCA.

1. Introduction

Cholangiocarcinoma (CCA), an adenocarcinoma of the epithelial cells of the biliary duct, is currently classified according to its anatomic locations as intrahepatic (ICCA) or extrahepatic (ECCA) cholangiocarcinoma [1]. The high incidence of CCA in Asian countries is associated with hepatic infections by liver flukes, while chronic biliary tract inflammation and cirrhosis are the prime causes of the disease found in America and Europe [2–4]. In general, the incidence of ECCA is higher than that of ICCA in western countries and Japan, while the opposite is true in eastern countries [4]. However, the incidence of ECCA in several countries has been reported to decrease markedly [4]. The differences between ICCA and ECCA were supported by different biological characteristics and sensitivity of CCA cells to chemotherapy [5]. The highest prevalence of CCA has been reported in the northeastern part of Thailand with age-standardized annual incidence rates of 115.0 per 100,000 in men and 52.7 per 100,000 in women [6]. In Thailand, an infection with *Opisthorchis viverrini* is the major risk factor associated with the development of both ICCA and ECCA [7]. On the other hand, CCA is associated with both hepatic infection (*Clonorchis sinensis*) and hepatolithiasis in Japan, Korea, Taiwan and China [8–10]. Taken together, both the biological characteristics and risk factors may be an important reason for the separation of the pathology and aggressiveness of ICCA and ECCA. In 2010, one of the seven established ICCA cell lines (KKU-M055, KKU-OCA17, KKU-M139, KKU-M214, KKU-100, KKU-M156 and KKU-M213) derived from Thai patients was shown to have a low degree of sensitivity to a commercial drug for CCA treatment (gemcitabine) [11]. This particular CCA cell line (KKU-M213) was found to have the most aggressive cells compared to other CCA cell lines [12]. However, the molecular mechanisms that control CCA cell

Abbreviations: CCA, cholangiocarcinoma; ICCA, intrahepatic cholangiocarcinoma; ECCA, extrahepatic cholangiocarcinoma; BCH, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid.

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behavior are largely unknown. Recently, the treatment for CCA remains a challenge because of the aggressive nature of the disease and its clinical symptoms that are silent until reaching an advanced stage [13]. At present, CCA patients are treated by surgical resection in combination with chemotherapy and radiation [14]. The surgery is, however, effective in only a few cases. In most cases of CCA patients who are inoperable, the survival rate of 5 years is less than 5% [15]. Thus, new diagnostic or prognostic markers as well as new molecular targeted therapies for CCA are urgently needed to improve survival rate.

L-type amino acid transporter 1 (LAT1) is the first isoform of system L amino acid transporter, which functions as an essential cellular component to provide large neutral amino acids across the cell membrane. LAT1 was isolated from C6 rat glioma cells by expression cloning. It is a 12-membrane-spanning protein, which mediates a Na^+-independent amino acid exchange and prefers large neutral amino acids for its substrates [16]. LAT1 forms a heterodimeric complex with the heavy chain of 4F2 antigen (4F2hc or CD98) via a disulfide bridge to recruit it to the plasma membrane [16,17]. The up-regulation of LAT1 and CD98 has been observed in several solid tumors such as brain, colon, lung, liver and skin [18]. Moreover, LAT1 and CD98 were also detected at high levels in several cancer cell lines such as leukemia cells, lung small cell carcinoma cells (RERF-LC-MA), uterine cervical carcinoma cells (HeLa) and bladder carcinoma cells (T24) [17]. Recently, LAT1 has been found to express at high level in biliary tract adenocarcinoma, closely correlated with lymphatic metastases [19]. The inhibition of system L amino acid transporters by using 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) significantly suppressed tumor growth and increased the sensitivity of the cancer to the therapeutic drugs gemcitabine and 5-FU [19]. These observations demonstrate that LAT1 plays an important role in the aggressiveness of cancer cells and may be an effective target for CCA treatment. However, the mechanisms of LAT1 function on the malignant phenotypes of CCA have not yet been reported, particularly in the highly aggressive cases with the unique cause of disease in Thai patients. Therefore, the present study aimed to investigate the role of LAT1 on the malignant phenotype of CCA and the molecular mechanisms underlying the inhibition of CCA malignancy by LAT1 knockdown in the CCA cell line derived from Thai patients, namely KKU-M213 cells.

Herein we showed that this cell line expressed all LAT isoforms and the expression of LAT1 was the highest. Suppression of LAT1 by shRNA knockdown inhibited leucine uptake and is concomitant with reduced cell migration and invasion. These effects were associated with the up-regulation of miRNA-7, which in turn suppressed 4F2hc–ERK1/2–p70S6K signaling.

2. Materials and methods

2.1. Cell culture, reagents and antibodies

Cholangiocarcinoma cells (KKU-M213) derived from Thai patients were obtained from Dr. Banchob Sripa (Liver Fluke and Cholangiocarcinoma Research Center, and Department of Pathology, Faculty of Medicine, Khon Kean University). Cells were cultured in Ham's F-12 medium supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) at 37 °C under a humidified 5% CO_2 incubator. The following reagents were used: TRIzol reagent (Invitrogen, Carlsbad, CA); BCA and Pierce Protein G Agarose (Pierce Biotechnology, Rockford, IL); Complete Mini EDTA-free and X-tremeGENE HP DNA transfection reagent (Roche, Mannheim, Germany); cDNA kit (Bio-Rad, Hercules, CA); SYBR kit (Biosystem, Woburn, MA); SuperSignal West Pico Chemiluminescent (Thermo Scientific, Rockford, MD); MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich); matrigel (BD Bioscience, San Jose, CA) and [^{14}C]L-leucine (Perkin Elmer, Boston, MA). The following antibodies were used for Western blot: anti-SLC7A5 (Abcam, ENG, UK); rabbit anti-CD98 (H-300) and mouse anti-CD98 (4F2) (Santa Cruz Biotechnology, Dallas, TX); anti-p70S6K and anti-phospho-p70S6K (T389) (Cell Signaling, Danvers, MA); anti-ERK1/2 and anti-phospho-ERK1/2 (Thr202/Tyr204) (BioVision, San Francisco, CA); anti-LC3 (Cell Signaling, Danvers, MA); anti-β-actin (Sigma-Aldrich); HRP goat anti-mouse IgG (H + L) and HRP goat anti-rabbit IgG (H + L) (Jackson ImmunoResearch Laboratories, West Grove, PA); goat anti-rabbit IgG (H + L) Alexa Fluor 594 and TO-PRO3 (Invitrogen).

2.2. LAT1 silencing

Two specific shRNAs targeting LAT1 (NM_003486.5) and shRNA negative control plasmids were generated using the pLentilox 3.7 expression vector. Oligonucleotides for shRNA are as follows: shRNA LAT1-319 sense strand, 5′-TGCACCACCATCTCCAAATCTTCAAGAGAGATTTGGAGATGGTG GTGCTTTTTC-3′ and antisense strand, 5′-TCGAGAAAAAAGCACCACCA TCTCCAAATCTCTCTTGAAGATTTGGAGATGGTGGTGCA-3′; shRNA LAT1-786 sense strand, 5′-TGTCACAGAGGAAATGATCATTCAAGAGATGATCAT TTCCTCTGTGACTTTTTTC-3′ and antisense strand, 5′-TCGAGAAAAAAG TCACAGAGGAAATGATCATCTCTTGAATGATCATTTCCTCTGTGACA-3′; and negative control sense strand, 5′-TACTATTATGCGGTGGCTAGTTCAAGAG ACTAGCCACCGCATAATAGTTTTTTTC-3′ and negative control antisense strand, 5′-TCGAGAAAAAAACTATTATGCGGTGGCTAGTCTCTTGAACTAGC CACCGCATAATAGTA-3′. To obtain lentiviral particles, the lentiviral plasmids were co-transfected with three lentiviral packaging plasmids (pMDLg/pRRE, pRSV-Rev and pMD2.G) (Addgene, Cambridge, MA) into HEK-293T cells by X-tremeGENE HP DNA transfection reagent (Roche) according to the manufacturer's protocol. The medium was collected at 48 h after transfection, filtered through 0.45 μm filters. To determine the lentiviral titer, cells were infected with a different dilution of lentiviral particles in the presence of 8 μg/ml polybrene. After 48 h, cells were trypsinized and GFP expression was analyzed by flow cytometry (FACS Aria II BD Biosciences). For experiments, KKU-M213 cells were transduced at a multiplicity of infection (MOI) = 1.

2.3. Cell proliferation and spreading assays

Cells were plated in 96-well plates in Ham's F-12 medium containing 10% FBS for 24 h. Cells were cultured for 8 days in the presence or absence of 20 mM BCH and the medium was changed every 3 days. To check the effect of LAT1 knockdown on cell proliferation, 48 h after lentiviral shRNA targeting LAT1 transduction, cells were plated in 96-well plates as mentioned above. Cell viability was determined by MTT assay. Cells were incubated with 0.5% MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution and incubated at 37 °C under a humidified 5% CO_2 incubator for 4 h. Then, the culture medium was removed and 100% DMSO was added before the measurement of absorbance at 590 nm by Multiskan GO Microplate Spectrophotometer (Thermo Scientific). The result was calculated as the % of cell viability. Cell spreading was carried out on 6-well plates, 24 h after plating to reach a confluent monolayer. A single scratch was created by using a sterile 200 μl micropipette tip and then incubated with or without 20 mM BCH in medium at 37 °C under a humidified 5% CO_2 incubator and observed under a light microscope after 18 and 24 h.

2.4. Cell migration and invasion assays

For an invasion assay, 48 h after lentiviral shRNA targeting LAT1 transduction cells were gently trypsinized and re-plated in the upper chamber (2.5×10^5 cells/chamber in serum-freed medium) of a transwell (6.5-mm diameter polycarbonate membrane with 8-μm pore size), coated with 30 μg of matrigel. The lower chamber was filled with 750 μL of Ham's F-12 media containing 10% FBS and incubated at 37 °C under a humidified 5% CO_2 incubator. After 12 h, non-invading cells in the upper chamber were removed by a cotton swab. The invaded cells that attached to the lower surface of the transwell membrane were fixed with 25% methanol for 20 min and stained with 0.5% crystal violet

for 1 h. Invaded cells were counted in random fields under a light microscope at 10× magnification. Cell migration assay were performed as the invasion assay, but without matrigel.

2.5. [^{14}C]L-leucine uptake measurement

Cells were seeded in 48-well plates and incubated at 37 °C under a humidified 5% CO_2 incubator. After 48 h of lentiviral shRNA targeting LAT1 transduction, cells were washed twice with D-PBS (137 mM NaCl, 3 mM KCl, 8 mM $Na_2HPO_4 \cdot 7H_20$, 1 mM KH_2PO_4, 0.5 mM $MgCl_2 \cdot 6H_2O$, 1 mM $CaCl_2 \cdot 2H_2O$ and 5.6 mM D-glucose and adjusted pH to 7.4 with NaOH). Cells were incubated at least 10 min in D-PBS at 37 °C, then further incubated with D-PBS containing [^{14}C]L-leucine (1 μM) in the presence or absence of BCH for 2 min. Uptake was stopped by removing the uptake solution and cells were washed with 500 μl of ice-cold D-PBS 3 times. Cells were solubilized with 10% SDS in 0.1 M NaOH and neutralized with 0.1 M HCl. The radio-activity was counted by scintillation spectrometry. Uptake rates were expressed as % of uptake compared to control cells. Three separate experiments were performed in order to confirm the reproducibility.

2.6. Immunofluorescence microscopy

KKU-M213 cells were seeded on glass cover slips in 24-well plates at the density of 2×10^5 cells/well and incubated at 37 °C under a humidified 5% CO_2 incubator. Cells were washed with cold PBS containing Ca^{2+}/Mg^{2+} (PBS^{++}), fixed with 100% cold methanol for 10 min and permeabilized with permeabilizing buffers (0.3% Triton X-100 and 0.3% BSA) for 30 min. Then, samples were incubated with rabbit anti-SLC7A5 and mouse anti-CD98 (4F2) antibodies for 24 h at 4 °C. Cells were washed with 0.3% Triton X-100 in PBS^{++} for 5 times and then incubated with Alexa Fluor® 488 goat anti-mouse IgG (H + L) or Alexa Fluor® 568 goat anti-rabbit IgG (H + L) diluted in 5% goat serum for 1 h. Cells were washed with 0.3% Triton X-100 in PBS^{++} for 5 times and stained with TO-PRO3 for 10 min at room temperature. The stained cell on cover slips were mounted with mounting medium (Vector Laboratories, Inc., Burlingame, CA) and visualized at room temperature under a confocal laser microscopy, Olympus FluoView FV10i (Olympus, Tokyo, Japan).

2.7. Total RNA extraction and quantitative real time PCR

Total RNAs were extracted using a Trizol reagent according to the manufacturer's instruction. The quality and quantity of total RNAs were determined using NanoDrop 2000C (Thermo Scientific). cDNAs were synthesized by using iScript™ cDNA synthesis kit according to the manufacturer's protocol and stored at −20 °C until further used. Quantitative real time PCR was performed on an equal amount of cDNA using KAPA SYBR® FAST quantitative PCR kit according to the instruction of the manufacturer with ABI PRISM7500 sequence detection system and analysis software (Applied Biosystems, Carlsbad, CA). The primers used were as follows: LAT1 (NM_003486.5), 5′-TTCGTCCAGATCGGGAAGGG TGA-3′ and 5′-TCCATAGGCAAAGAGGCCGCT-3′; LAT2 (NM_012244.2), 5′-GGGAGCCCTCTGCTATGCTGAAC-3′ and 5′-GAACCCAGCCAGTCCTCC GAAGA-3′; LAT3 (NM_003627.4), 5′-GCTGGTTGGCAGTGCCTGCT-3′ and 5′-GCCATTCAGGGACAGCGCCA-3′; LAT4 (NM_152346.1), 5′-AACC AAACGCTCTCTCCGTGCT-3′ and 5′-ACCGAAGGTCGCCGAACATGT-3′; 4F2hc (NM_002394.4), 5′-GGCGCAGAAGTGGTGGCACA-3′ and 5′-CCCC TTCAGACCCGCCAGGT-3′; ASCT2 (NM_001145145), 5′-TGGTACGAAA ATGTGGGCA-3′ and 5′-GTGCCCCAGCAGGCAGCACA-3′ and β-actin (NM_001101.3), 5′-TACCCTGGCATTGCCGACAGGA-3′ and 5′-TACCCT GGCATTGCCGACAGGA-3′. The mRNA level of each gene was normalized to mRNA level of β-actin.

2.8. MicroRNA extraction and detection

Total RNAs were extracted from cultured cells by using miRNAeasy RNA isolation kit (Qiagen, Hilden, Germany). cDNAs were synthesized by using miScript II RT kit (Qiagen) according to the manufacturer's protocol and stored at −20 °C. miRNA was analyzed by realtime PCR using miScript primer assay with miScript SYBR Green PCR kits (Qiagen) according to the manufacturer's protocol. The level of miR-7 expression was normalized to the expression of a small RNA (RNU6-2).

2.9. Western blot analysis and immunoprecipitation

KKU-M213 cells were plated at a cell density of 1×10^6 cells per petri dish (100 mm) and incubated at 37 °C under a humidified 5% CO_2 incubator. Cells were harvested and lysed with a modified radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF and protease inhibitor cocktail). For kinase protein, cells were starved with serum-free Ham's F-12 medium for 16–18 h. After 20 min of incubation on ice, cells were centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatants were collected and protein concentration was measured by the BCA (Bicinchoninic acid) method. Equal amount of proteins were mixed with Laemmli buffer and heated at 95 °C for 5 min. Proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and subsequently transferred to a nitrocellulose membrane by electro-blotting. Membranes were incubated overnight at 4 °C with anti-SLC7A5, anti-CD98 (H-300), anti-ERK1/2, anti-phospho-ERK1/2 (Thr202/Tyr204), anti-p70S6K, anti-phospho-p70S6K (T389) and anti-β-actin antibodies. Membranes were washed and incubated with HRP goat anti-rabbit secondary antibody. The signals were detected using the enhanced SuperSignal West Pico Chemiluminescent. For co-immunoprecipitation, 500 μg of lysates were incubated with 50 μl of Protein G Agarose slurry for 1 h and centrifuged for 5 min at 2500 ×*g*. The collected supernatants were incubated overnight with mouse anti-CD98 (4F2) or normal mouse IgG (as a negative control) at 4 °C. Protein G agarose slurry (100 μl) was added and incubated for 2 h at room temperature. Agarose beads were then washed with IP buffer (25 mM Tris, 150 mM NaCl, pH 7.2) for 5 times and once with water. To elute the complex, 50 μl of Laemmli buffer was added, incubated at 95 °C for 5 min and centrifuged at 2500 ×*g*. The supernatants were collected and separated by SDS-PAGE. Membranes were incubated overnight at 4 °C with rabbit anti-SLC7A5 and rabbit anti-CD98 (H-300) antibodies.

2.10. Statistical analysis

All data were expressed as means and standard error of means (means ± S.E.M.). The statistical analysis was analyzed by using the statistical software package, GraphPad Prism version 5.0. The statistically significant differences among groups were compared using one-way analysis of variance (ANOVA) followed by Tukey–Kramer post hoc test. Unpaired Student's *t* test was used when applicable. Statistical significance was considered when $p < 0.05$.

3. Results

3.1. Expression of system L amino acid transporters in human cholangiocarcinoma cells

In the present study, the cholangiocarcinoma cells from Thai CCA patients, KKU-M213, was used to examine the expression of system L amino acid transporters. The mRNA expressions of system L amino acid transporters (LAT1, LAT2, LAT3, and LAT4), 4F2hc and ASCT2 were determined by quantitative real time PCR. KKU-M213 cells showed high mRNA levels of LAT1 and 4F2hc of the system L1 associating protein (Fig. 1A). Interestingly, the expression of system L2 amino

acid transporter (LAT3) and ASCT2, an obligate amino acid exchanger of LAT1, were also detected, although their expressions were lower than that of LAT1. On the other hand, the expression levels of LAT2 and LAT4 were barely detectible in KKU-M213 cells. The localization of LAT1 and 4F2ch were investigated by confocal microscopic analysis. We found that both LAT1 and 4F2hc were predominantly co-localized on the plasma membrane of KKU-M213 cells (Fig. 1B). The interaction of LAT1 and 4F2hc was further examined by coimmunoprecipitation. Results showed that LAT1 could be coimmunoprecipitated with 4F2hc in KKU-M213 cells (Fig. 1C). We next confirmed the association of LAT1 and 4F2hc in these cells by separation on SDS-PAGE in the presence [DTT(+)] and absence [DTT(−)] of DTT followed by staining with anti-SLC7A5 and anti-4F2hc (anti-CD98) antibodies. As shown in Fig. 1D, the 125 kDa bands corresponding to the LAT1-4F2hc heterodimer complex were detected in KKU-M213 cells in the absence of DTT [DTT(−)]. However, under a reducing condition [DTT(+)], LAT1 and 4F2hc were detected at 38 kDa and 85 kDa bands corresponding to the original sizes of LAT1 and 4F2hc, respectively. These results indicate that LAT1 and 4F2hc proteins co-exist as a heterodimer complex linked via a disulfide bond and is expressed on the plasma membrane of KKU-M213 cells.

3.2. Inhibition of system L amino acid transporters suppresses KKU-M213 cell growth and migration

As leucine uptake is mediated by system L amino acid transporter, to investigate its function in KKU-M213 cells, we evaluated [^{14}C]L-leucine uptake in the presence or absence of BCH, a competitive inhibitor of system L. As shown in Fig. 2A, the uptake was significantly inhibited by 100 μM BCH (approximately 50–60%) ($p < 0.001$). The result indicates

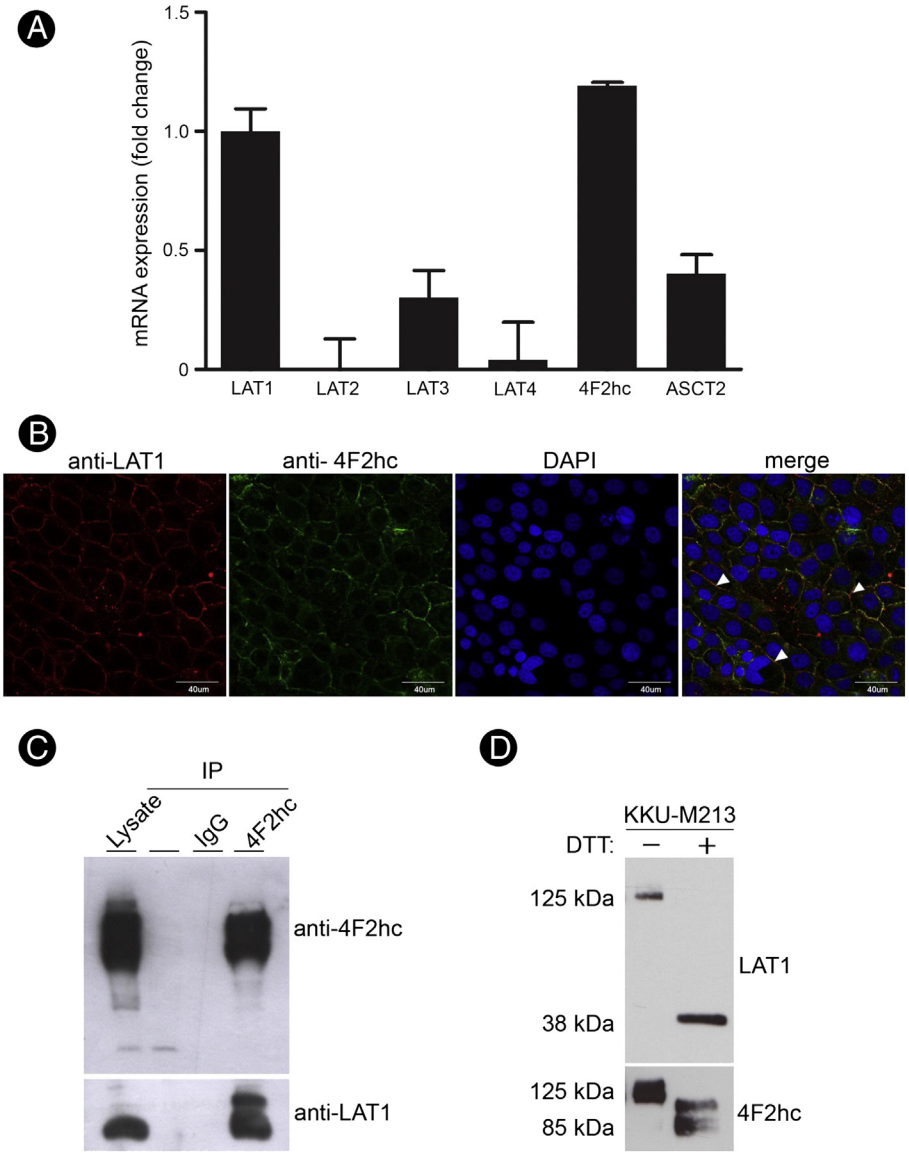

Fig. 1. Characterization of system L amino acid transporters in human cholangiocarcinoma cells. (A) Expression of system L1 and L2 amino acid transporters in CCA cells. Total RNAs were extracted from KKU-M213 cells and analyzed for LAT1, LAT2, LAT3, LAT4, 4F2hc and ASCT2 expressions by quantitative real time PCR. Data are expressed as fold change compared with the expression level of LAT1 and presented as means ± S.E.M. (n = 3). The expression of β-actin was used for normalization. (B) Co-localization of LAT1 and 4F2hc on the plasma membrane. KKU-M213 cells were fixed and stained with anti-SLC7A5 (LAT1; red) and anti-CD98 (4F2hc; green) antibodies. TOPRO-3 was used as a nuclear marker (blue). Arrow heads indicate co-localization of LAT1 and 4F2hc. Scale bar = 40 μm. (C) LAT1 interacts with 4F2hc in CCA cells. Cell lysates were immunoprecipitated with anti-CD98 antibody and normal mouse IgG. The immunoprecipitated samples were analyzed by western blotting with the indicated antibodies. (D) Association between LAT1 and 4F2hc by a disulfide bond in CCA cells. Western blot analysis was performed on KKU-M213 cell lysates in the presence [DTT(+)] or absence [DTT(−)] of DTT using anti-SLC7A5 and anti-4F2hc antibodies.

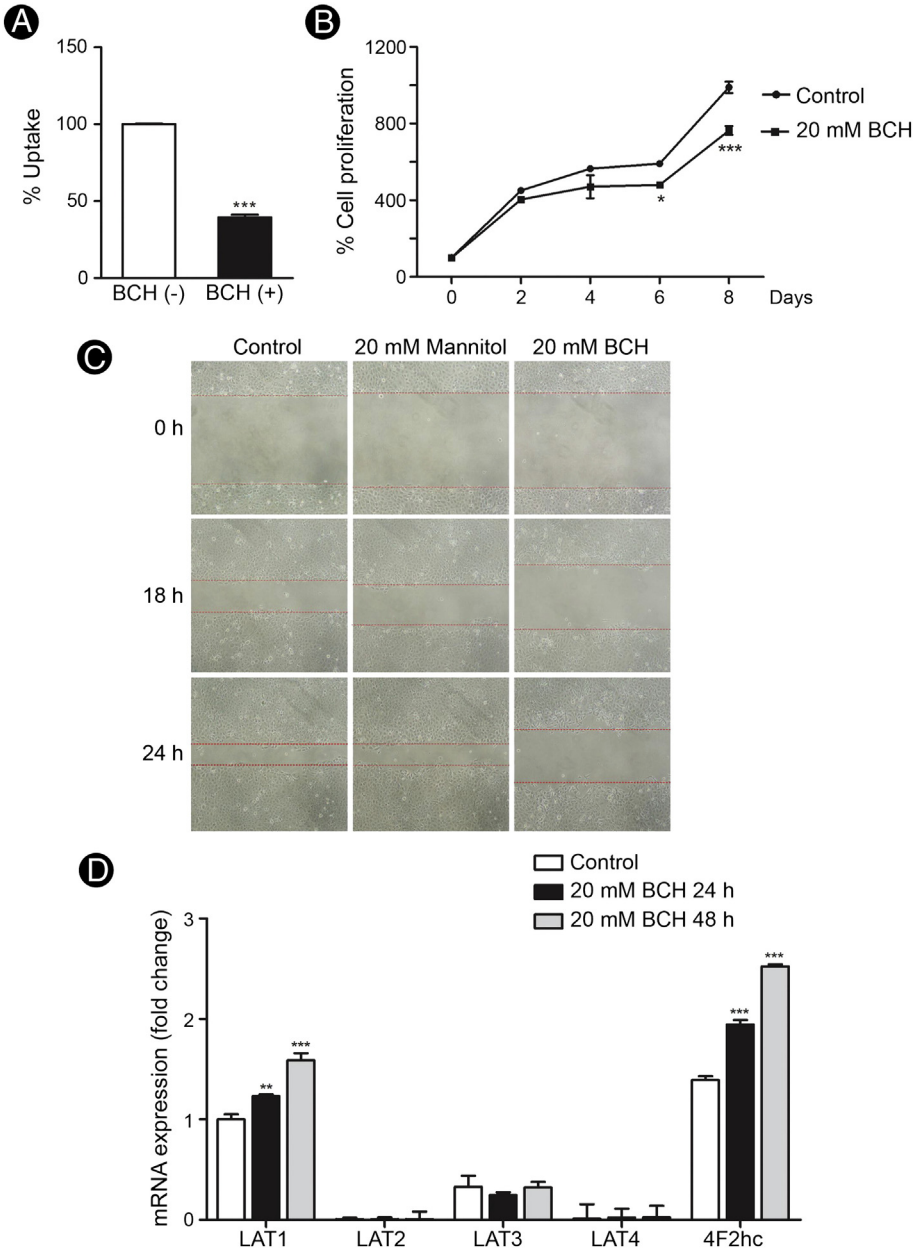

Fig. 2. The effect of system L inhibitor (BCH) treatment on human cholangiocarcinoma cells. (A) Inhibition of leucine transport by BCH. The [^{14}C]L-leucine (1 μM) uptake in KKU-M213 cells was measured in the presence or absence of 100 μM BCH for 2 min. The results are means ± S.E.M. (n = 3) and presented as % of uptake (Student's *t* test); ***, $p < 0.001$ compared with control (absence of BCH). (B) The effect of BCH on CCA cell proliferation. KKU-M213 cells were incubated with 20 mM BCH and cell proliferation was measured by using an MTT assay every 2 days for 8 days. The results are means ± S.E.M. (n = 3) and presented as % of cell proliferation (ANOVA); *, $p < 0.05$; ***, $p < 0.001$ compared with control (absence of BCH). (C) Inhibition of CCA cell migration by BCH. KKU-M213 cells were plated on 6-well plates to reach a confluent monolayer. A single scratch was created using a micropipette tip and then incubated with medium in the absence or presence of 20 mM BCH for 18 and 24 h. (D) The effect of BCH on system L amino acid transporter expressions in CCA cells. Total RNAs were extracted from KKU-M213 cells treated with 20 mM BCH for 24 and 48 h and analyzed for LAT1, LAT2, LAT3, LAT4, and 4F2hc expressions by quantitative real time PCR. The mRNA expressions of LAT1 and 4F2hc were significantly increased in a time-dependent manner. The results are means ± S.E.M. (n = 3) and presented as fold change. The expression of β-actin was used for normalization (ANOVA); **, $p < 0.01$; ***, $p < 0.001$ compared with control (absence of BCH).

that the uptake of leucine, an essential amino acid, into CCA cells is mediated by system L amino acid transporters. We next investigated the impact of system L on CCA cell proliferation and migration. BCH was used to inhibit the amino acid uptake activity of system L in KKU-M213 cells and cell proliferation was measured by MTT assay. Treatment with 20 mM BCH for 4 days did not significantly affect cell proliferation. However, cell proliferation was slightly but significantly reduced after BCH treatment for 6 ($p < 0.05$) and 8 ($p < 0.001$) days (Fig. 2B). Although BCH showed less effect on cell proliferation, it markedly suppressed cell migration. As illustrated in Fig. 2C, KKU-M213 cell migration was suppressed as early as 18 h after incubation with 20 mM BCH compared to control (20 mM mannitol treated cells). These data suggest the important roles of system L on malignant phenotypes of KKU-M213 cells.

In addition, an up-regulation of LAT1 was seen in BCH treatment. Thus, by 24 and 48 h after treatment, the mRNA levels of LAT1 and 4F2hc were significantly increased (Fig. 2D). In contrast, treatment with BCH has no effect on the expressions of LAT2, LAT3 and LAT4.

These results suggest that the inhibition of system L amino acid transporter by BCH has a feedback effect to up-regulate amino acid transporter expression.

3.3. LAT1 is the system L amino acid transporter that mediates amino acid uptake into cholangiocarcinoma cells

Since BCH inhibited all members of system L amino acid transporters, we next tested whether the inhibition of cancer cell phenotypes by BCH involves LAT1 function. LAT1 gene knockdown in KKU-M213 cells was conducted by transient transduction with lentiviral shRNAs targeting LAT1 (shRNA LAT1-319 and shRNA LAT1-786). After lentiviral infection, the LAT1 expression was determined by quantitative real time PCR and western blot analysis. As shown in Fig. 3A, the LAT1 mRNA expressions were significantly reduced in KKU-M213 cells infected with both shRNA LAT1-319 and shRNA LAT1-786 compared to shRNA negative control. Consistent with the mRNA data, the expressions of LAT1 protein were reduced after transduction with both lentiviral shRNA

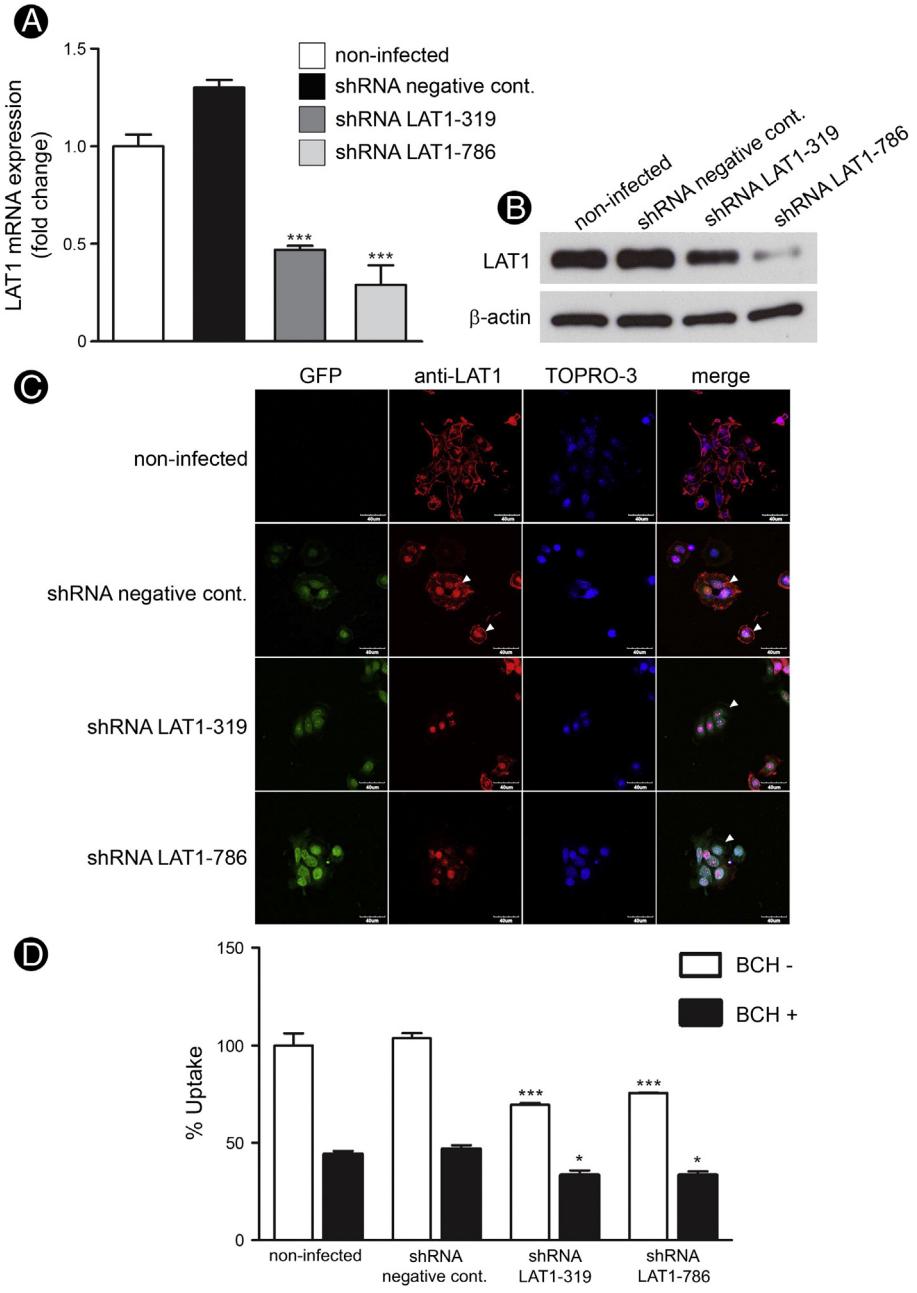

Fig. 3. Silencing of L-type amino acid transporter 1 (LAT1) increases the sensitivity of CCA cells to BCH. (A) Inhibition of LAT1 mRNA expression by lentiviral shRNA targeting LAT1 in KKU-M213 cells. Total RNAs were extracted from KKU-M213 cells after being infected with lentiviral shRNA targeting LAT1 (shRNA LAT1-319 and shRNA LAT1-786) or shRNA negative control for 48 h and analyzed for LAT1 expression by quantitative real time PCR. The results are means ± S.E.M. (n = 3) and presented as fold change (ANOVA); ***, $p < 0.001$ compared with shRNA negative control infected cells. (B–C) Infection with lentiviral shRNA targeted LAT1 suppresses LAT1 protein expression and localization. KKU-M213 cells were infected with lentiviral shRNA targeting LAT1 or shRNA negative control for 48 h and harvested for immunoblotting using anti-SLC7A5 (LAT1) and anti-β-actin antibodies (B). For cellular localization, KKU-M213 cells were infected with lentiviral shRNA targeting LAT1 or shRNA negative control for 48 h. Cells were fixed and immunostained for LAT1 using anti-SLC7A5 (LAT1) antibody (red). TOPRO-3 was used for nuclear marker (blue). The GFP signals represent cells that were infected by lentiviral shRNA plasmids. The plasma membrane localization of LAT1 (arrows head) was reduced in LAT1 knockdown cells, scale bar = 40 μm (C). (D) LAT1 knockdown suppresses leucine transport activity in KKU-M213 cells. [^{14}C]L-leucine (1 μM) uptake was performed in KKU-M213 cells infected with either shRNA negative control or shRNA targeted LAT1 in the presence or absence of 100 μM BCH for 2 min. The results are presented as means ± S.E.M. (n = 3) (ANOVA); ***, $p < 0.001$ compared with shRNA negative control infected cells; *, $p < 0.05$ compared with shRNA negative control infected cells and treated with BCH.

constructs. However, shRNA LAT1-786 was more effective than shRNA LAT1-319 (Fig. 3B). The reduction of LAT1 mRNA and protein expressions was further confirmed by confocal microscopy. Indeed, the localization of LAT1 on the plasma membrane was significantly reduced in KKU-M213 cells infected with both lentiviral shRNAs targeting LAT1 (Fig. 3C). In order to examine the effect of LAT1 knockdown on amino acid transport, [^{14}C]L-leucine (1 μM) uptake was performed in KKU-M213 cells infected with lentiviral shRNAs in the presence or absence of 100 μM BCH. As shown in Fig. 3D, [^{14}C]L-leucine uptake was significantly decreased in LAT1 knockdown cells compared to shRNA negative control cells. Taken together, these results indicate that leucine uptake into KKU-M213 cells is mediated by LAT1. Suppression of cell

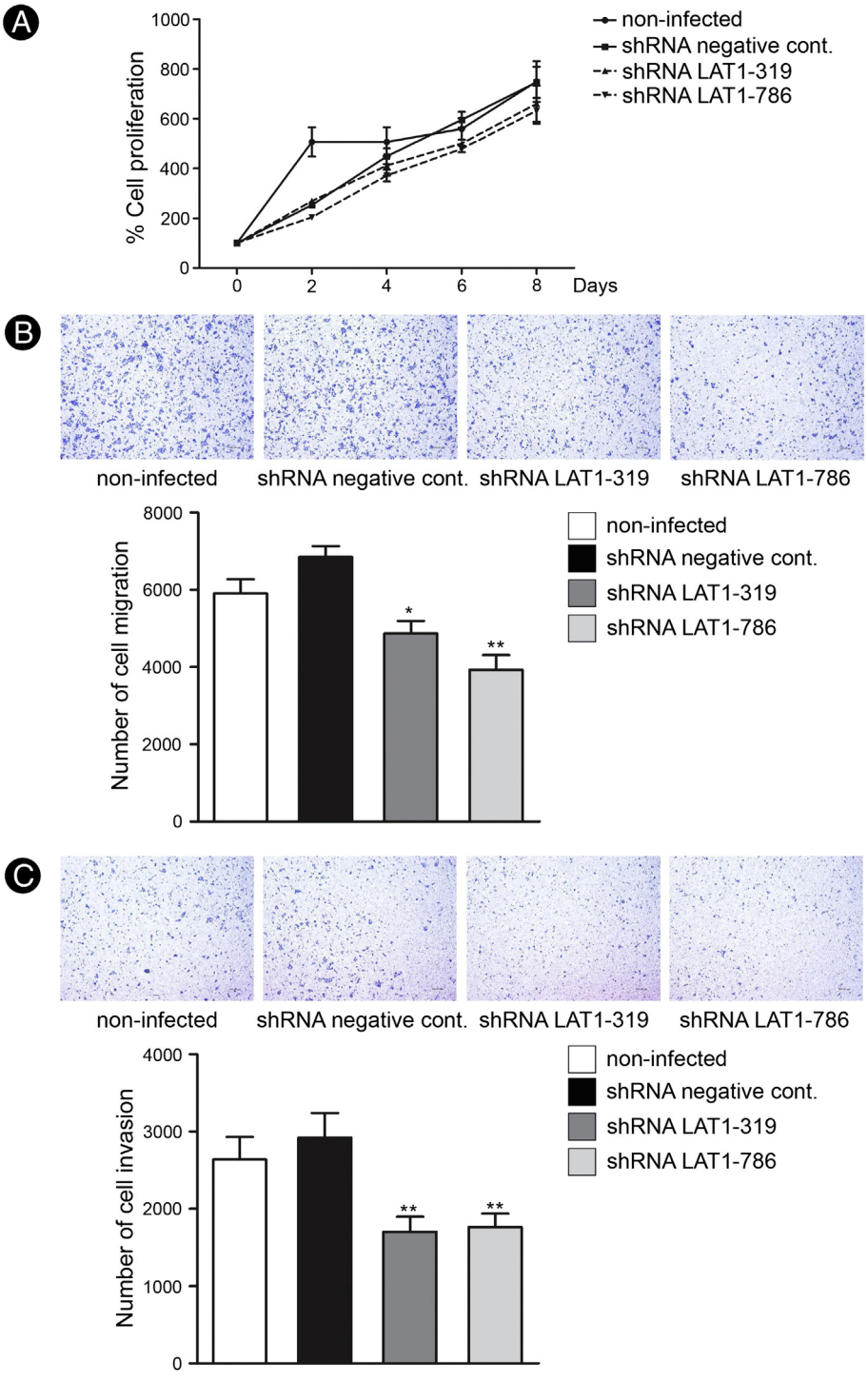

Fig. 4. LAT1 knockdown suppresses malignant phenotypes of KKU-M213 cells. (A) Suppression of LAT1 expression has no effect on cell proliferation. Infected lentiviral shRNA targeting LAT1 (shRNA LAT1-319 and shRNA LAT1-786) or shRNA negative control KKU-M213 cells were plated on 96-well plates. Cell proliferation was determined by MTT assay every 2 days for 8 days. (B–C) Suppression of CCA cell migration and invasion by LAT1 knockdown. Infected lentiviral shRNA targeting LAT1 (shRNA LAT1-319 and shRNA LAT1-786) or shRNA negative control cells were plated in the upper chamber of a trans-well coated with or without matrigel for invasion and migration assay, respectively. The lower chamber was filled with complete medium containing 10% FBS. After 12 h of incubation, numbers of migrating (B) and invading (C) cells were counted. Bar graphs show the number of migrating and invading cells and are presented as means ± S.E.M. (n = 5) (ANOVA); *, $p < 0.05$; **, $p < 0.01$ compared with shRNA negative control infected cells.

proliferation and migration by BCH might, in part, be due to the inhibition of LAT1-mediated essential amino acid uptake into KKU-M213 cells.

3.4. LAT1 regulates CCA cell migration and invasion

We further examined the role of LAT1 on CCA cell proliferation, migration and invasion. The role of LAT1 on CCA cell proliferation was determined after transduction of lentiviral shRNA into KKU-M213 cells by MTT assay at days 2, 4, 6 and 8. Fig. 4A shows that suppression of LAT1 expression did not affect cell proliferation compared to the negative control infected cells. This was not due to the inefficiency of LAT1 knockdown after transient transduction of lentiviral shRNA because the mRNA expression of LAT1 was reduced by 60–70% at day 8 (data not shown) compared to negative control cells. Amino acid deprivation is known to trigger autophagy that provides an alternative energy source for their survival. We, therefore, tested whether limitation of amino acid by downregulation of LAT1 would activate autophagy. Indeed, amino acid deprivation by incubating cells with Earle's balanced salt solution (EBSS) for 2 h induced autophagy as demonstrated by the accumulation of autophagosome-incorporated LC3-II (Fig. S1). On the other hand, limitation of amino acid availability by shRNA-mediated downregulation of LAT1 did not activate autophagy in KKU-M213 cells (Fig. S1). Next, we investigated the functions of LAT1 on the malignant progression of CCA cells, i.e. cell migration and invasion. By 48 h after lentiviral shRNA infection, the number of migrated and invaded cells were examined by migration and invasion assays, respectively. As shown in Fig. 4B, cell migration was significantly decreased in KKU-M213 cells infected with lentiviral shRNA LAT1-319 (30%) and shRNA LAT1-786 (40%), respectively, compared to the negative control. Likewise, cell invasion was significantly decreased in LAT1 knockdown cells (approximately 40%) (Fig. 4C). The results indicate that LAT1 plays an important role in cell migration and invasion, but has less effect on KKU-M213 cell proliferation.

3.5. Inhibition of 4F2hc expression in LAT1 knockdown cells

As shown in Fig. 2D, the inhibition of system L function by BCH treatment increased the expressions of LAT1 and 4F2hc. We, therefore, investigated the expression of other isoforms of system L amino acid transporters (LAT2, LAT3 and LAT4), 4F2hc and ASCT2 in LAT1 knockdown CCA cells by quantitative real time PCR. Interestingly, silencing LAT1 did not significantly alter the mRNA expressions of LAT2, LAT3, LAT4 and ASCT2 (Fig. S2), indicating that there are no compensatory responses by other isoforms of system L after LAT1 knockdown. In contrast, both mRNA and protein expressions of 4F2hc in LAT1 knockdown cells were significantly decreased (Fig. 5A and B). Furthermore, the expression of 4F2hc on the plasma membrane was also reduced (arrows head) compared to the negative control (Fig. 5C). The molecular mechanism by which 4F2hc expression is reduced is presently not well understood. However, a previous study showed that microRNA-7 is a modulator molecule that regulates 4F2hc gene expression [20]. Therefore, the expression of microRNA-7 in LAT1 knockdown KKU-M213 cells was determined. We found that, indeed, microRNA-7 was significantly increased in KKU-M213 cells transiently infected with lentiviral shRNA (shRNA LAT1-786) compared to the negative control (Fig. 5D). These results suggest that the suppression of CCA cell invasion and migration may be due to the inhibition of 4F2hc by microRNA-7 in LAT1 knockdown cells.

3.6. Effect of LAT1 suppression on ERK1/2 and p70S6K signaling

The complex of 4F2hc-integrin β1-ERK1/2 is required for cancer cell motility and invasion [21]. In addition, the activation of ERK1/2 directly phosphorylates p70S6K, which is the downstream mediator of mTOR signaling pathway that is involved in cancer progression [22]. Therefore, we determined whether inhibition of CCA cell invasion and migration by LAT1 knockdown would affect ERK1/2 and p70S6K activity. As shown in Fig. 6, the activity of ERK1/2 and p70S6K were significantly reduced as illustrated by the reduction of the levels of ERK1/2 and p70S6K phosphorylation in KKU-M213 cells transiently infected with lentiviral shRNAs targeting LAT1. These results indicate that an inhibition of malignant phenotypes by LAT1 knockdown is partly mediated by the suppression of ERK1/2 and p70S6K activity, the 4F2hc-related signaling.

4. Discussion

Cholangiocarcinoma (CCA) is the second most common primary malignancy of the liver and highly prevalent in the northeastern part of Thailand [23]. CCA is extremely invasive and often metastasizes to other organs and tissues with poor prognosis. Currently, chemotherapeutic treatment of CCA in Thailand is ineffective. Therefore, understanding of tumor carcinogenesis and identification of new molecular therapeutic targets or biomarkers that would predict clinical outcome is urgently needed. This would benefit clinicians in developing effective therapeutic strategies for CCA. LAT1, a system L1 amino acid transporter, has been shown to be expressed at high level in various types of tumors including biliary tract cancer [19]. However, the expression of LAT1 and insight into its molecular mechanism involved in tumorigenicity for CCA cells particularly those derived from Thai patients are largely unknown. In the present study, we showed for the first time that this cell line expressed all LAT isoforms (LAT1, LAT2, LAT3, and LAT4) and the expression of LAT1 was the highest. Suppression of LAT1 either by BCH or by LAT1 knockdown inhibited leucine uptake and cell migration, but had no effect on cell proliferation. These effects were associated with up-regulation of miRNA-7 and suppression 4F2hc-ERK1/2-p70S6K signaling.

Cancer cells require a large supply of nutrients to meet their demands for rapid growth and cell division. Thus, up-regulation of amino acid transporters especially the system L amino acid transporters that supply large neutral amino acids including many essential amino acids has been reported in several types of cancer [18]. Our findings that KKU-M213 cells highly expressed LAT1 and its associated protein 4F2hc, and that these proteins formed a heterodimer complex on the plasma membrane are consistent with the previous reports in various types of cancer including biliary tract cancer [18,19]. In contrast, LAT2, the second isoform of amino acid transport system L1 which is abundant and plays a role in the growth and proliferation of normal cells [24], was barely detectible in KKU-M213 cells. On the other hand, the system L2 amino acid transporters (LAT3 and LAT4), which are the sodium-independent plasma membrane transporters with low affinity for large neutral amino acids [25,26], were both expressed in KKU-M213 cells. However, LAT4, the second isoform of system L2 amino acid transporter, was expressed at a very low level whereas LAT3 was the second most abundant in KKU-M213 cells. The latter finding was in contrast with the previous report also in biliary duct cancer [19]. The discrepancy may be due, in part, to the fact that the KKU-M213 cell line was developed from Thai patients diagnosed with intrahepatic carcinoma [27] while the HuCC-T1 cell line was established from patients with moderately differentiated adenocarcinoma originating from the intrahepatic biliary tract in Japan [28]. Development of CCA in Thai patients is highly correlated with *O. viverrini* infection which is different from those found in Japan [7–10]. LAT3 was identified from a human hepatocarcinoma-derived cell line (FLC4) and found to be expressed in the pancreas, liver and skeletal muscle cells [25]. The role of LAT3 in cancer cell growth and proliferation is, however, still unclear. Recently, Wang and colleagues demonstrated the expression of LAT3 in prostate cancer cell lines (LNCaP and PC-3) and its inhibition by shRNA led to a significant decrease in cell growth [29]. Further studies are therefore required to precisely understand the roles of system L2 amino transporter in CCA cells.

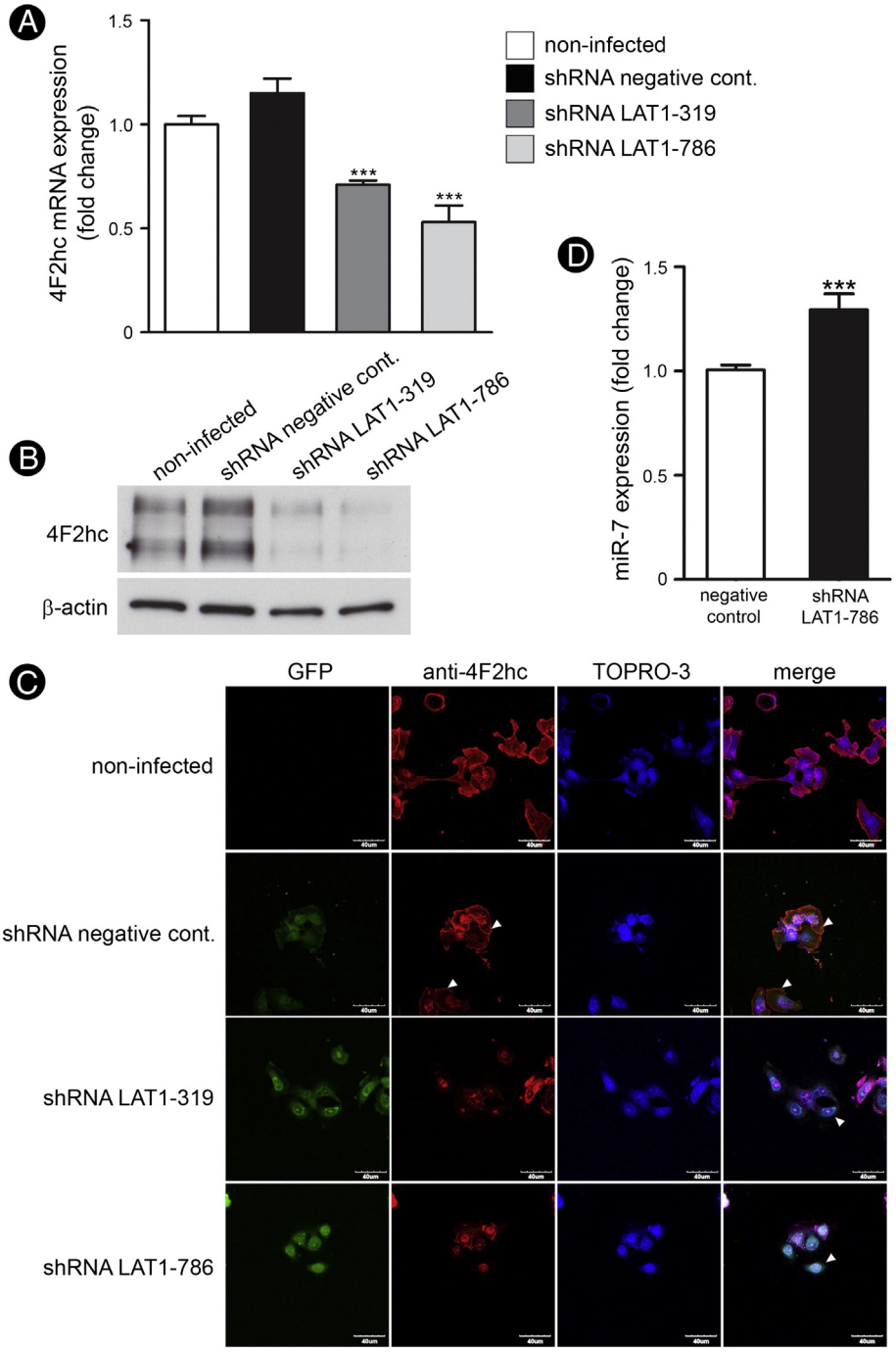

Fig. 5. Reduction of 4F2hc in LAT1 knockdown KKU-M213 cells. (A) Suppression of 4F2hc mRNA in LAT1 knockdown CCA cells. KKU-M213 cells were infected with lentiviral shRNA targeting LAT1 (shRNA LAT1-319 and shRNA LAT1-786) or shRNA negative control for 48 h. Total RNAs were extracted for quantitative real time PCR of 4F2hc. The results are means ± S.E.M. (n = 3) and presented as fold change (ANOVA); ***, $p < 0.001$ compared with shRNA negative control infected cells. (B) Reduction of 4F2hc protein expression in LAT1 knockdown CCA cells. Protein lysates were prepared from KKU-M213 cells 48 h after infection with lentiviral shRNA targeting LAT1 (shRNA LAT1-319 and shRNA LAT1-786) or shRNA negative control and analyzed by Western blotting using anti-4F2hc and anti-β-actin antibodies. (C) Reduction of 4F2hc protein on the plasma membrane in LAT1 knockdown CCA cells. Forty-eight hours after lentiviral infection, KKU-M213 cells were fixed and immunostained for 4F2hc using anti-4F2hc antibody. TOPRO-3 was used as a nuclear marker (blue). The GFP signals represent cells that were infected by lentiviral shRNA plasmids. The localization of 4F2hc on the plasma membrane (arrows head) was reduced in LAT1 knockdown cells, scale bar = 40 μm. (D) Up-regulation of miR-7 in LAT1 knockdown KKU-M213 cells. The expression of miR-7 was measured by quantitative real time PCR of total RNAs extracted from LAT1 knockdown KKU-M213 cells. The miR-7 expression was normalized by expression of RNU6-2 as an internal control gene. The results are means ± S.E.M. (n = 3) and presented as fold change (Student's *t* test); ***, $p < 0.001$ compared with shRNA negative control infected cells.

In the present study, the expressions of LAT1 and 4F2hc were increased in KKU-M213 cells after treatment with BCH suggesting a compensatory response of system L amino acid transporters. In agreement with this notion, a previous study in mouse trophoblast cells showed that LAT1 mRNA expression was up-regulated by BCH [30]. One important mechanism to maintain intracellular amino acid level is the up-regulation of amino acid transporter induced by ATF4 (Activating transcription factor 4), which responds to nutrient deprivation by increasing the transcription of transporters such as LAT1, CAT1, ASCT2 and 4F2hc [29,31,32]. Similarly, the inhibition of leucine

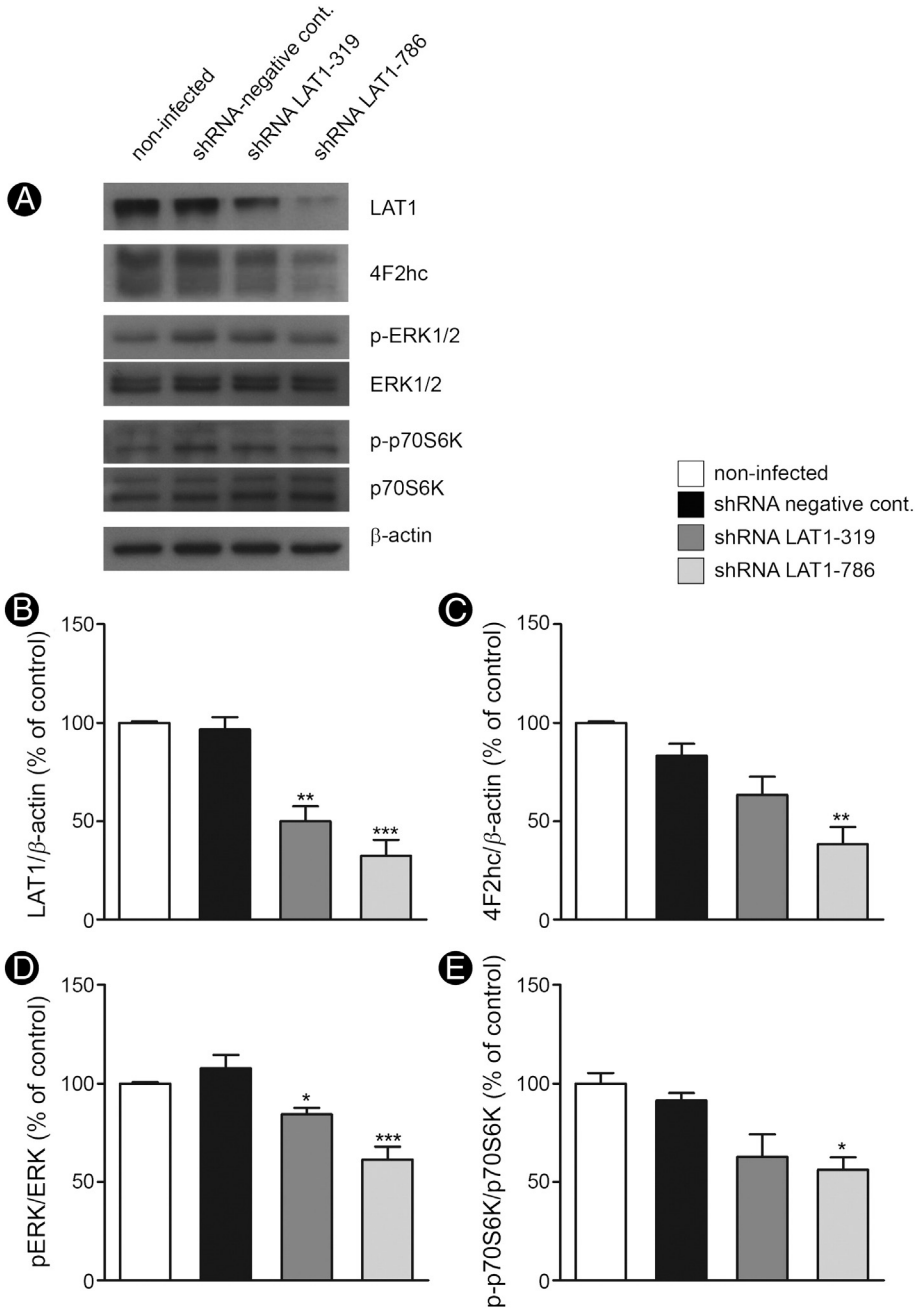

Fig. 6. Suppression of ERK1/2 and p70S6K activities by shRNA targeted LAT1. Total cell lysates extracted from KKU-M213 cells after infection with lentiviral shRNA targeting LAT1 (shRNA LAT1-319 and shRNA LAT1-786) or shRNA negative control for 48 h were analyzed by western blotting using the indicated antibodies (A). Bar graphs show the mean normalized densitometry values and the corresponding standard deviations for LAT1 (B), 4F2hc (C), ERK1/2 (D), and p70S6K (E). Data are means ± S.E.M. (n = 3) and presented as % of control (ANOVA); *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with shRNA negative control infected cells.

transport by BCH resulted in the up-regulation of ATF4 in prostate cancer cells [29]. Therefore, the effect of BCH on the up-regulation of LAT1 and 4F2hc transcription in KKU-M213 cells may be due to the activation of ATF4 activity.

Although BCH is widely used to study the LAT1 function, it is known to be an inhibitor for all sodium-independent system L amino acid transporters including LAT1, LAT2, LAT3, and LAT4 [16,24–26]. Thus, we investigated the impact of LAT1 on CCA using lentiviral shRNA targeting LAT1. It has been reported that knockdown of LAT1 expression suppressed trophoblast cell invasion [30]. Also, inhibition of LAT1 expression by shRNA reduced leucine uptake and proliferation of prostate cancer cells (LNCaP and PC-3) [29]. However, the role of LAT1 on cell migration, proliferation and invasion has not yet been reported in CCA cells. In this study, we found that reduction of LAT1 expression by shRNA caused significant reductions of KKU-M213 cell migration and invasion, but not proliferation. The lack of effect of LAT1-knockdown on proliferation has also been shown in human ovarian cancer cells [33]. Of note, our results that the expression levels of LAT2, LAT3, LAT4 and ASCT2 were not affected in LAT1 knockdown cells suggest that the lack of effect on cell proliferation is not due to the compensation by the up-regulation of other isoforms of system L amino acid transporters. In addition, the lack of this effect on CCA cell survival is not due to the alternative supply of amino acids from self-digested organelles since the limitation of amino acid availability by LAT1 shRNA failed

to activate autophagy. On the other hand, the results indicate that other biological factors may be involved in LAT1-independent moderation of CCA cell proliferation. Further experiments are required to completely understand the precise molecular mechanisms by which the malignant phenotypes mediated by LAT1 are regulated in different types of cancer cells.

Although the effects of LAT1 knockdown on 4F2hc protein expression have previously been reported in many types of cells, our work showed for the first time that mRNA expression of 4F2hc was altered in the LAT1 knockdown CCA cells. In HeLa cells, the expression of 4F2hc protein was decreased when LAT1 was targeted by RNAi [34]. Likewise, both disruption and truncation of LAT1 resulted in decreased 4F2hc protein expression in chicken DT40 cells [35]. However, it is not clear how suppression of LAT1 affected 4F2hc expression in the above studies. The protein 4F2hc is a type II glycosylated protein that belongs to the SLC3 family including SLC3A1/rBAT and SLC3A2/4F2hc [36]. The role of a light chain subunit on its heavy chain partner trafficking has been demonstrated. Deletion of the C-terminus of $b^{0,+}$AT (light chain subunit of SLA3A1/rBAT) has no effect on the heterodimer formation with rBAT. On the other hand, the transport function was lost due to the failure of the plasma membrane targeting of the rBAT–$b^{0,+}$AT heterodimer complex [37]. The role of its light chains on the transcription regulation of their heavy chains is, however, largely unknown. Recently, post-transcriptional regulation of 4F2hc has been reported. Nguyen and co-workers demonstrated that miR-7 down-regulated 4F2hc expression by directly targeting the 3′-UTR of 4F2hc mRNA and modulated β1-integrin–laminin-1 interactions [20]. Interestingly, it has been reported that miR-7 functions as a tumor suppressor gene in several human cancers including lung cancer [38], breast cancer [39], and hepatocellular carcinoma [40]. Overexpression of miR-7 inhibited tumor growth and metastasis [38–40]. Surprisingly, we found that miR-7 expression was significantly increased in LAT1 knockdown KKU-M213 cells. Thus, our data suggest that modulation of 4F2hc expression by LAT1 in KKU-M213 cells may be mediated through miR-7. Therefore, it is of interest to further study the mechanisms by which LAT1 regulates miR-7 expression and the role of miR-7 on the expression of other target genes in CCA cells. In contrast, the increases in LAT1 and 4F2hc expression after BCH treatment had no effect on the miR-7 expression (Fig. S3). In agreement with this notion, a previous study in transgenic mice overexpressing CD98 found that, under basal conditions, CD98 overexpression did not affect the expression of miRNAs [41]. Although the precise mechanism remains unclear, our results suggest that the up-regulation of LAT1 and 4F2hc in KKU-M213 cells in response to limited availability of amino acid by treatment with BCH may not involve the regulation by miR-7.

The suppression of CCA cell migration and invasion by silencing LAT1 may involve 4F2hc-related signaling. It has been shown that 4F2hc interacts with β1-integrin and is involved in integrin-dependent signaling to promote tumor cell survival and proliferation [42]. Deletion of 4F2hc resulted in the accumulation of β1 integrin in the cytoplasm and impaired integrin signaling [43]. The β1 integrin–4F2hc complex that activates FAK, Rac and Akt signaling has been found in many tumors and transformed cells and contributes to cellular behaviors leading to tumorigenesis [42]. In the mesangial cells (MCs), α1β1 integrin-mediated collagen matrix reorganization regulates cell migration via the ERK1/2 signaling pathway [44]. ERK1/2 is known as an important molecular signaling molecule necessary for several cellular functions such as proliferation, differentiation, migration, and invasion [45]. Although ERK1/2 activities have been found both in normal and tumor cells, sustained elevation of ERK1/2 activity was observed only in tumor cells. In contrast, its activity declined rapidly in normal cells [46]. In our study, we found that the phosphorylation of ERK1/2 was reduced in LAT1 knockdown KKU-M213 cells suggesting the involvement of ERK1/2 signaling in suppression of cell migration and invasion. This is consistent with the previous report that HGF regulated invasiveness of two independent CCA cell lines (HuCCA1 and KKU-M213) by different signaling pathways, i.e. PI3K was a common pathway in both CCA cell lines whereas ERK1/2 activation was crucial for KKU-M213 cell invasion [47]. Furthermore, we also found that p70S6K activity was reduced in LAT1 knockdown cells. P70S6K is a downstream effector of mTOR signaling pathway. It is phosphorylated by mTOR after amino acid stimulation and also directly phosphorylated by ERK1/2 [22]. The activation of p70S6K promotes protein synthesis, which plays crucial roles in cell proliferation, migration and invasion [48]. Collectively, our results suggest that silencing of LAT1 in CCA cells resulted in the reduction of 4F2hc-associated and mTOR signaling pathways and subsequently inhibited cell migration and invasion.

5. Conclusion

In summary, our study demonstrated that LAT1 and its associated protein, 4F2hc, are up-regulated in KKU-M213 cells derived from human intrahepatic bile duct cancer. To our knowledge, we showed for the first time that miR-7 expression was regulated by LAT1. Up-regulation of miR-7 resulted in down-regulation of 4F2hc expression, which, in turn, affected KKU-M213 cell invasion and migration through the inhibition of the 4F2hc–ERK1/2–p70S6K signaling pathway. Since 4F2hc plays significant roles in normal cell functions and development, systemic interfering with 4F2hc expression and/or function may not be a good strategy for cancer treatment. Therefore, targeted knockdown of LAT1, which is highly expressed in cancer cells, would specifically suppress tumor growth and metastasis or enhance the sensitivity of cancer cells to chemotherapeutic drugs with less serious adverse effects to normal cells. Collectively, our study provides the scientific rationale for using LAT1 as new molecular targets to develop new strategies for cholangiocarcinoma treatment.

Conflict of interest

The authors have no conflicts of interest to disclose.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2014.04.002.

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Proteomics profiling of cholangiocarcinoma exosomes: A potential role of oncogenic protein transferring in cancer progression

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ABSTRACT

Cholangiocarcinoma (CCA), a common primary malignant tumor of bile duct epithelia, is highly prevalent in Asian countries and unresponsive to chemotherapeutic drugs. Thus, a newly recognized biological entity for early diagnosis and treatment is highly needed. Exosomes are small membrane bound vesicles found in body fluids and released by most cell types including cancer cells. The vesicles contain specific subset of proteins and nucleic acids corresponding to cell types and play essential roles in pathophysiological processes. The present study aimed to assess the protein profiles of CCA-derived exosomes and their potential roles. We have isolated exosomes from CCA cells namely KKU-M213 and KKU-100 derived from Thai patients and their roles were investigated by incubation with normal human cholangiocyte (H69) cells. Exosomes were internalized into H69 cells and had no effects on viability or proliferation of the host cells. Interestingly, the exosomes from KKU-M213 cells only induced migration and invasion of H69 cells. Proteomic analysis of the exosomes from KKU-M213 cells disclosed multiple cancer related proteins that are not present in H69 exosomes. Consistent with the protein profile, treatment with KKU-M213 exosomes induced β-catenin and reduced E-cadherin expressions in H69 cells. Collectively, our results suggest that a direct cell-to-cell transfer of oncogenic proteins via exosomal pathway may be a novel mechanism for CCA progression and metastasis.

1. Introduction

Cholangiocarcinoma (CCA), a severe neoplasm of biliary tract epithelia [1], is the second most common primary hepatic tumor globally [2]. CCA is often enigmatic, characterized by poor prognosis, and unresponsive to chemotherapeutic agents [3]. This type of cancer represents 10–25% of primary liver cancers worldwide and is highly prevalent in Asian countries including Thailand [4]. It responds poorly to the presently available therapies [5] and surgical resection is the only mean to extend lifespan for no more than 5 years [6]. It is, therefore, considered to be an incurable and rapidly lethal malignancy. In view of the lack of a valid early stage detection method and potential curative treatment, an effective early detection and/or an alternative therapeutic strategy with high sensitivity to specific targets is urgently needed.

Exosomes are small (40–100 nm in diameter) membrane bound vesicles that are initially formed within the endosomal compartment and secreted upon fusion of the limiting membrane of multi-vesicular bodies (MVBs) with plasma membrane [7]. These homogeneous vesicles are released by most of cell types including cancer cells and are considered as messengers of intercellular communications [8]. Biochemical and proteomic analysis of exosomes reveal that, besides a common set of membrane and cytosolic molecules, these vesicles contain cell and cell state specific cargos of proteins, mRNAs, and miRNAs that characterize their functional activities [9]. Of particular interest, tumor cells exhibit enhanced production of exosomes. The exact function and the need of such high amount of exosomes in malignant cells are not clearly understood. Recent investigations suggest the roles in cell-to-cell communication, tumor–stroma interaction, antigen presentation and establishment of tumor microenvironments that potentially uphold cancer progression at different steps [10]. Although exosomes from a variety of cell types have been isolated and characterized and their functions have been previously reported, the presence and the role of exosomes in CCA carcinogenesis are presently not known. We hypothesized that CCA cells may

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manufacture exosomes that reinforce CCA development. The present piece of work investigated whether CCA cells produce exosomes and what are their potential roles on malignant phenotypes. We found that KKU-M213 cells, an aggressive human CCA cell line, released exosomes in culture media with basic characteristics similar to those isolated from other sources [11]. A comparative proteomics approach between KKU-M213 and human cholangiocyte (H69) exosomes disclosed vast differences in their active protein contents. Furthermore, exosomes from KKU-M213 cells increased motility and altered cell adhesion related protein expressions in recipient H69 cells. Our results indicate that exosomes may play a role on CCA pathogenesis and identification of their cargo proteins may serve as a biomarker for early detection and thus might enlighten new treatment strategies.

2. Materials and methods

2.1. Reagents and antibodies

Ham's F12 nutrient mixture, DMEM, DMEM/F-12 media and antibiotic–antimycotic were purchased from Invitrogen (CA, USA). Fetal bovine serum (FBS), protease and phosphatase inhibitor, adenine, insulin, epinephrine, 3,3′,5 Triiodothyronine, apo-transferrin, epidermal growth factor and hydrocortisone were obtained from Sigma-Aldrich Co. (MO, USA). RIPA cell lysis buffer and SuperSignal West Pico Chemiluminescent Substrate were purchased from Thermo Scientific (Cramlington, UK). Anti-flotillin-1 and anti-E-cadherin primary antibodies were purchased from BD Biosciences (CA, USA). Anti-GSK3β and anti-phospho-GSK3β (Ser-9) were obtained from Cell Signal Technologies (MA, USA). Anti-β-catenin and anti-CD81 (B-11) antibodies were from Santa Cruz Biotechnology, Inc. (TX, USA). Anti-TSG101[4A10] and anti-CD63 were from abcam (Cambridge, UK). Anti-β-actin was form Sigma-Aldrich Co. (MO, USA). Trans-well chambers were obtained from Corning Life Sciences (MA, USA). All other reagents were of the highest analytical grades available and unless otherwise stated were purchased from Sigma-Aldrich (MO, USA).

2.2. Cell culture

KKU-100 and KKU-M213, the CCA cell lines established from primary tumors of *Opisthorchiasis*-associated Thai CCA patients [12], were kindly provided by Dr. Banchob Sripa. KKU-100 is poorly differentiated adenocarcinoma cell line with compact polygonal to spindle shape [12] whereas KKU-M213 is well-differentiated adenocarcinoma cell line [13,14]. These cells were cultured in Ham's F12 nutrient media, supplemented with 10% heat inactivated FBS and 1% antibiotic–antimycotic, at 37 °C in a humidified 5% CO_2 incubator. A human cholangiocyte cell line (H69) immortalized with the simian virus 40 (SV40) large T antigen was obtained from Dr. Gregory J. Gores, Mayo Clinic College of Medicine and cultured in DMEM/Ham-F12 media containing 10% FBS. Although immortal, this cell line is non-malignant; *in vitro*, it does not display anchorage independent growth and, *in vivo*, it does not produce tumors in nude mice [15].

2.3. Exosome isolation

CCA or H69 cells were plated in 75 cm^2 culture flasks. Cells were cultured in conditioned medium depleted of contaminating vesicles and protein aggregates by overnight centrifugation at 110,000 ×*g*. Culture supernatants were collected 48 h after changing the medium and exosomes were purified by differential centrifugation as described previously [16]. Briefly, culture supernatant was collected and first centrifuged 300 ×*g* at 4 °C for 10 min to remove lifted cells followed by centrifugation at 1,200 ×*g* for 10 min and 10,000 ×*g* at 4 °C for 30 min to remove cell debris. The resultant supernatant was subjected to filtration on 0.22 μm pore filters, followed by ultracentrifugation (Beckman Coulter Inc.; CA, USA) at 110,000 ×*g* for 70 min. The resulting pellets were re-suspended in chilled PBS, pooled, and again ultra-centrifuged at 110,000 ×*g* for 70 min at 4 °C. The final pellet of exosomes was re-suspended in 50–100 μl PBS, aliquoted and stored at −80 °C until use.

2.4. Electron microscopy

For electron microscopic (EM) observation of exosomes, pellets obtained after centrifugation at 110,000 ×*g* were fixed in 4% paraformaldehyde (PFA) (1:1 ratio) for 10 min and loaded on Formvar/carbon-coated EM grids by floating the grid over small drops of PBS containing fixed exosomes. The exosomes were then post fixed in 1% glutaraldehyde for 5 min and contrasted successively in freshly prepared 2% uranyl acetate, pH 7, and 2% methylcellulose/0.4% uranyl acetate, pH 4. Observations were made with a FEI Technai G^2 electron microscope (The Netherlands) equipped with a thermionic tungsten filament and operated at an acceleration voltage of 120 kV. Images were taken with a cooled slow-scan CCD camera at a magnification of 50,000×.

2.5. Migration and invasion assay

Cell migration and invasion assays were performed using trans-well chambers with polycarbonate inserts containing 8 μm pores, as described elsewhere [17]. Briefly, H69 cells were detached from culture plates by gentle pipetting. Cells (1×10^5 cells) were mixed with PBS or 200 μg/ml exosomes from either H69 cells or CCA cells, placed inside the upper chamber, and maintained in serum- and growth factor-freed medium for 48 h. For an invasion assay, a thin layer of matrigel was layered on top of the insert in the upper chambers. Cells, incubated at 37 °C in a humidified 5% CO_2 incubator, were allowed to migrate or invade for 48 h to the lower chamber containing 750 μl culture medium supplemented with 20% exosome-freed FBS as chemo-attractant. After incubation, non-migrated and non-invaded cells in the upper chamber were completely removed using cotton swabs. Cells that had migrated or invaded across the porous membrane were fixed in 25% methanol for 30 min, stained with DAPI (1 μg/ml) for 5 min in darkness or 0.5% crystal violet and washed 3 times. The membranes were carefully removed and mounted on slides. The nuclei of migrated and invaded cells were observed under a confocal fluorescence microscope and photographed. Cells were automatically counted using image J software. The background was subtracted using rolling ball feature, jointed cells were separated by pixel mapping and parameter of the software was set to exclude particles of smaller or bigger than the average nuclear size to minimize erroneous counting.

2.6. Cell viability and cell proliferation assay

Cell viability was measured by MTT assay. H69 cells were plated in 96-well plates for 24 h. The supernatants were withdrawn from sub-confluent cultures, the cells were washed with serum-freed medium and incubated with PBS or 200 μg/ml of exosomes from either H69 or CCA cells, in serum- and growth factor-freed medium for 12, 24 or 48 h. Culture supernatants were then carefully removed and MTT (0.5 mg/ml) solutions were added and incubated at 37 °C, 5% CO_2 incubator for 4 h. Then, MTT solutions were removed and 100 μl DMSO was added to dissolve the formazan crystals before measurement at an absorbance of 590 nm by Multiskan micro plate reader (Thermo Scientific; Cramlington, UK). The result was calculated as % of cell viability. Cell proliferation upon exosomes treatment was further analyzed by staining Ki67 protein in growing cells. H69 cells were cultured on cover slips in 24-well culture plates. After 24 h, supernatants were withdrawn from sub-confluent cultures, the cells were washed with serum-freed medium and incubated with PBS or 200 μg/ml of exosomes from either H69 or CCA cells, in serum- and growth factor-freed medium. After culture, supernatants were carefully removed, cells were washed twice in PBS and fixed in 4% PFA. Cells were then blocked in 10% BSA for 1 h, washed in PBS and incubated with cocktail antibody solution in 1% BSA containing anti-ki67 and anti-actin antibodies for 2 h at room

temperature. Then, the cells were washed thrice and incubated with fluorescent tagged secondary antibodies for another 1 h at room temperature in darkness. Cells were then washed and nuclei were counter stained with DAPI (0.5 mg/ml) for 5 min, mounted on glass slides and visualized under a confocal microscope (Fluoview fv10i, Olympus).

2.7. SDS-PAGE and nano LC/MS/MS

Exosome samples (30 μg) from H69 and KKU-M213 cells were re-suspended in NuPAGE SDS sample buffer and separated under reducing conditions on NuPAGE 3–8% gradient precast tris-acetate SDS–polyacrylamide gel (Life Technologies; NY, USA). Gels were stained with Coomassie brilliant blue g-250 gel staining solution (Bio-Rad; CA, USA), and de-stained in ultrapure LC/MS grade water (Fisher Scientific; Loughborough, UK). Each gel lane was trimmed in pieces and de-stained in 50% acetonitrile (in 25 mM NH_4HCO_3) until colorless. Freshly prepared 10 mM dithiothreitol (in 25 mM NH_4HCO_3) was added to reduce the proteins for 15 min at 60 °C. The gel pieces were cooled down to room temperature and freshly prepared 55 mM iodoacetamide (in 25 mM NH_4HCO_3) were added to alkylate the proteins for 30 min at room temperature in the dark. Thereafter, solutions were removed and acetonitrile was added to dehydrate gel pieces. The gel pieces were allowed to dry

Table 1
Protein identification from H69 and KKU-M213-derived exosomes.

No	Accession no.	Protein	emPAI	
			H69	KKU-M213
1	CLH1_HUMAN	Clathrin heavy chain 1	0.49 ± 0.03	0.41 ± 0.04
2	FINC_HUMAN	Fibronectin	0.2 ± 0.05	0.1 ± 0.03
3	HSP7C_HUMAN	Heat shock cognate 71 kDa protein	0.75 ± 0.05	0.17 ± 0.1
4	PDC6I_HUMAN	Programmed cell death 6-interacting protein	0.52 ± 0.17	0.7 ± 0.11
5	UBIQ_HUMAN	Ubiquitin	6.06 ± 1.24	3.77 ± 1.52
6	EF1A1_HUMAN	Elongation factor 1-alpha 1	0.43 ± 0.01	NI
7	HS90B_HUMAN	Heat shock protein HSP 90-beta	0.19 ± 0.28	NI
8	ACTB_HUMAN	Actin, cytoplasmic 1	0.29 ± 0.00	0.29 ± 0.00
9	TERA_HUMAN	Transitional endoplasmic reticulum ATPase	0.18 ± 0.00	0.18 ± 0.00
10	HSP76_HUMAN	Heat shock 70 kDa protein 6	0.17 ± 0.03	NI
11	ANXA2_HUMAN	Annexin A2	0.32 ± 0.00	0.32 ± 0.00
12	ACTBL_HUMAN	Beta-actin-like protein 2	0.19 ± 0.00	0.19 ± 0.00
13	ALBU_HUMAN	Serum albumin	0.17 ± 0.00	0.3 ± 0.00
14	TS101_HUMAN	Tumor susceptibility gene 101 protein	0.28 ± 0.07	NI
15	LRRK2_HUMAN	Leucine-rich repeat serine/threonine-protein kinase 2	NQ	NI
16	ATD3B_HUMAN	ATPase family AAA domain-containing protein 3B	NQ	NI
17	RRBP1_HUMAN	Ribosome-binding protein 1	NQ	NI
18	LG3BP_HUMAN	Galectin-3-binding protein	NI	1.56 ± 0.23
19	FPRP_HUMAN	Prostaglandin F2 receptor negative regulator	NI	0.68 ± 0.07
20	4F2_HUMAN	4F2 cell-surface antigen heavy chain	NI	0.89 ± 0.04
21	MVP_HUMAN	Major vault protein	NI	0.44 ± 0.06
22	ITB1_HUMAN	Integrin beta-1	NI	0.39 ± 0.1
23	AT1A1_HUMAN	Sodium/potassium-transporting ATPase subunit alpha-1	NI	0.21 ± 0.13
24	TSP1_HUMAN	Thrombospondin-1	NI	0.18 ± 0.02
25	K1199_HUMAN	Protein KIAA1199	NI	0.15 ± 0.04
26	TACD2_HUMAN	Tumor-associated calcium signal transducer 2	NI	0.49 ± 0.09
27	EF2_HUMAN	Elongation factor 2	NI	0.16 ± 0.01
28	1A01_HUMAN	HLA class I histocompatibility antigen, A-1 alpha chain	NI	0.55 ± 0.07
29	G3P_HUMAN	Glyceraldehyde-3-phosphate dehydrogenase	NI	0.35 ± 0.09
30	ITB4_HUMAN	Integrin beta-4	NI	0.07 ± 0.00
31	LAMC1_HUMAN	Laminin subunit gamma-1	NI	0.06 ± 0.00
32	1B55_HUMAN	HLA class I histocompatibility antigen, B-55 alpha chain	NI	0.3 ± 0.07
33	TPP2_HUMAN	Tripeptidyl-peptidase 2	NI	0.14 ± 0.04
34	TINAL_HUMAN	Tubulointerstitial nephritis antigen-like	NI	0.23 ± 0.01
35	HSP71_HUMAN	Heat shock 70 kDa protein 1A/1B	NI	0.11 ± 0.00
36	ANXA1_HUMAN	Annexin A1	NI	0.2 ± 0.09
37	HS90A_HUMAN	Heat shock protein HSP 90-alpha	NI	0.14 ± 0.06
38	BASI_HUMAN	Basigin	NI	0.29 ± 0.08
39	UBA1_HUMAN	Ubiquitin-like modifier-activating enzyme 1	NI	0.1 ± 0.00
40	IMB1_HUMAN	Importin subunit beta-1	NI	0.08 ± 0.00
41	IGSF8_HUMAN	Immunoglobulin superfamily member 8	NI	0.18 ± 0.00
42	ITA3_HUMAN	Integrin alpha-3	NI	0.1 ± 0.00
43	ITA2_HUMAN	Integrin alpha-2	NI	0.09 ± 0.02
44	TBB2C_HUMAN	Tubulin beta-2C chain	NI	0.16 ± 0.11
45	APOB_HUMAN	Apolipoprotein B-100	NI	0.01 ± 0.00
46	VTNC_HUMAN	Vitronectin	NI	0.14 ± 0.06
47	MFGM_HUMAN	Lactadherin	NI	0.09 ± 0.02
48	ITA6_HUMAN	Integrin alpha-6	NI	0.06 ± 0.00
49	EPCAM_HUMAN	Epithelial cell adhesion molecule	NI	0.23 ± 0.1
50	KPYM_HUMAN	Pyruvate kinase isozymes M1/M2	NI	0.13 ± 0.07
51	TBA1B_HUMAN	Tubulin alpha-1B chain	NI	0.15 ± 0.08
52	PLAK_HUMAN	Junction plakoglobin	NI	0.09 ± 0.00
53	ARRD1_HUMAN	Arrestin domain-containing protein 1	NI	0.17 ± 0.00
54	AAAT_HUMAN	Neutral amino acid transporter B(0)	NI	0.14±
55	PGBM_HUMAN	Basement membrane-specific heparan sulfate proteoglycan core protein	NI	0.01±
56	PB1_HUMAN	Protein polybromo-1	NI	NQ
57	CENPE_HUMAN	Centromere-associated protein E	NI	NQ

NI: No identificationand NQ: no quantification by emPAI.

completely in a fume hood. Trypsin solution (0.01 mg/ml) was added to digest the gel pieces and incubated at 37 °C overnight. The peptides were extracted by adding acetonitrile (10 mg/ml in 50% HPLC grade acetonitrile, 0.1% TFA) and subsequently concentrated using a concentrator. The samples were stored at −20 °C prior to mass spectrometric analysis. Each tryptic digested fractions were re-suspended in 0.1% formic acid containing 2% acetonitrile and analyzed by MicroToF Q II mass spectrometer (Bruker; Bremen, Germany). The front end of the mass spectrometer was coupled with an Ultimate 3000 nano-LC system (Dionex; Surrey, UK). The separation was done at a flow rate of 300 nl/min. Mobile phase A (2% (v/v) acetonitrile, 0.1% (v/v) formic acid in HPLC grade water) and mobile phase B (0.1% (v/v) formic acid in HPLC grade acetonitrile) were used to establish 45 min gradient. The gradient started with 10 min 2–10% B, followed by 33 min 10–40% B, ramped rapidly (1 min) 40–95% B and maintained at 95% B for 1 min. The eluent was sprayed and ionized in the nano-electrospray source of the mass spectrometer. Data were acquired using Hystar software. The MS and MS/MS spectra were acquired in mass range of m/z 400–2000 m/z 50–1500, respectively.

2.8. Analysis of LC/MS/MS data

The mass spectrometric data was smoothed, centroided and converted into a mascot generic file (.mgf) using data analysis software version 4.0. Mascot v.2.3.0 (Matrix Science, London, UK) was used to search data against Swissprot_57.15 (total sequences is 515203) human protein database using trypsin with one possible missed cleavage allowed at 1. The mass tolerances for precursor and fragment ions were set to 1.2 Da and 0.6 Da, respectively. The peptide charge was selected as 2^+, 3^+ and 4^+. Oxidations of methionine and carbamidomethylation of cysteine were set as variable modifications. To reduce false positive identification, only peptides scored above 20 were reported in this study and each identified protein contained at least 2 peptides. Due to the same amount of proteins loaded into SDS-PAGE,

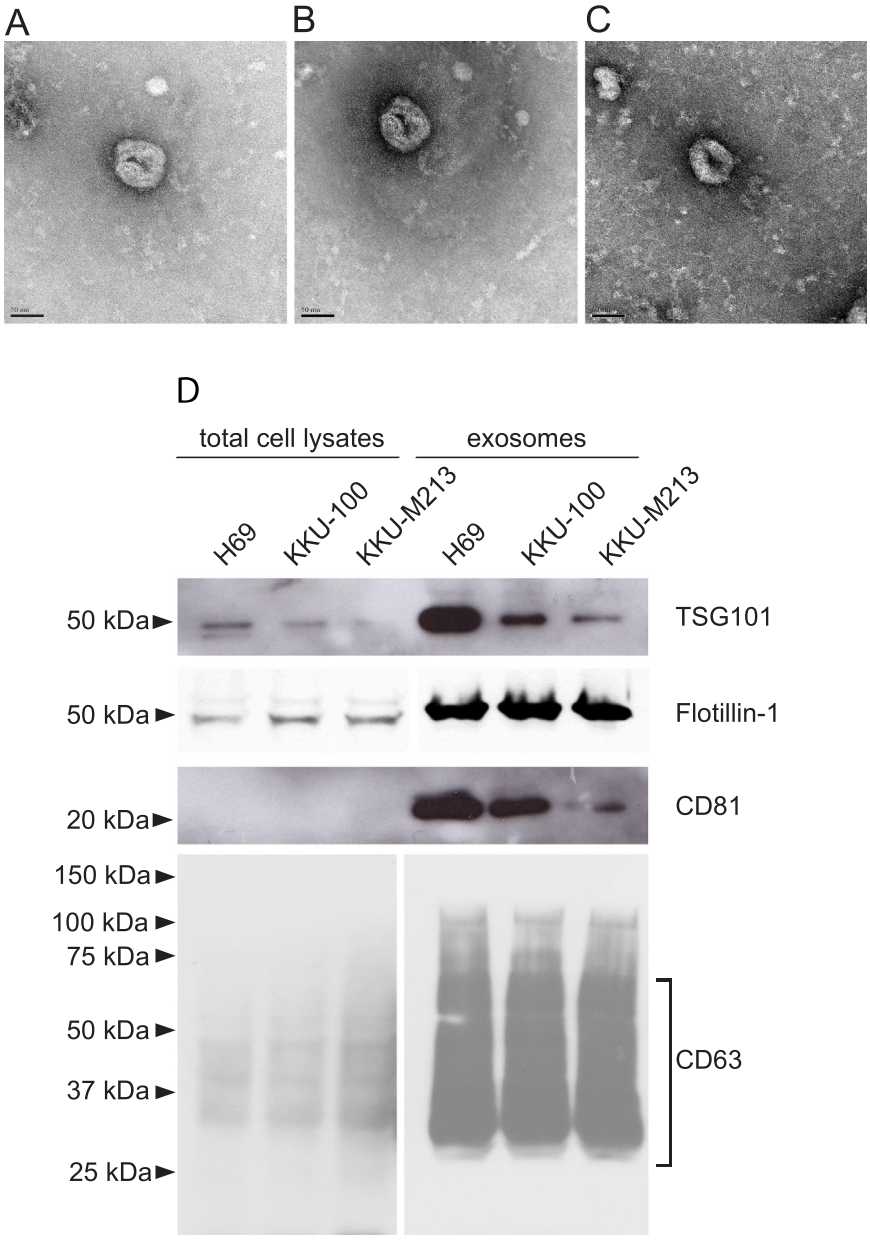

Fig. 1. Characterization of exosomes purified from CCA cell lines. Transmission electron microscopic images of exosomes isolated from (A) H69, (B) KKU-100, and (C) KKU-M213 cell lines. (D) Western blot analysis of exosome marker proteins. Total cell lysates and isolated exosome fractions (50 μg for CD63, 25 μg for flotillin-1, 1 μg for TSG101 and CD81) from three different cell lines were separated by 10% SDS-PAGE followed by Western blotting with specific antibodies. Bars = 50 nm.

quantification information of H69 and KKU-M213 was performed using exponentially modified protein abundance index (emPAI) provided by the Mascot. The emPAI value is based on an equation shown below:

$$\text{emPAI} = 10^{\frac{N_{observed}}{N_{observable}}} - 1$$

where $N_{observed}$ is the number of experimentally observed peptides and $N_{observable}$ is the calculated number of observable peptides for each protein [18]. The success of this label freed quantitative approach has been shown in many studies. Indeed, secretomes of *Aspergillus fumigatus* were profiled at different temperatures [19]. Moreover, the mannose-binding proteins (MBPs) from the normal donor and hepatocellular carcinoma (HCC) patient sera were also quantified by emPAI [20]. All emPAI values in Table 1 are means of three MS analysis. Protein class and pathway categorization were carried out using Panther classification system available at http://www.pantherdb.org/. The functional annotation of identified proteins was mainly retrieved from the Swissprot database (http://www.uniprot.org/).

2.9. Western blot analysis

H69 cells were cultured on 6-well culture plates. After treatment with exosomes in serum- and growth factor-freed medium for the desired period, cells were washed thrice with chilled PBS. Cells were then harvested, lysed with radio-immuno-precipitation assay (RIPA) cell lysis buffer (50 mM Tris–HCL pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF) containing protease and phosphatase inhibitors. Lysates were cleared by centrifugation at 14,000 ×*g* for 20 min at 4 °C. The supernatant fractions were used for Western blotting. The protein concentrations were measured using BCA (Bicinchoninic acid) protein assay kit (Thermo Fisher; MA, USA). Equal amount of protein extracts from different treatment sets were mixed with Laemmli sample buffer and heated at 95 °C for 5 min. The proteins were resolved by 10% SDS-PAGE and transferred onto PVDF membrane at a constant voltage of 100 V at 4 °C for 2 h. Membranes were blocked with 5% non-fat dry milk for 1 h at room temperature and probed with indicated antibodies overnight at 4 °C. Membranes were then washed thrice and incubated with HRP conjugated anti-mouse or anti-rabbit secondary antibody for 1 h at room temperature and signals were detected using enhanced SuperSignal West Pico Chemiluminescent (Thermo Scientific; MA, USA).

2.10. Confocal fluorescence microscopy

H69 cells were plated on cover slips in 12-well plates for 24 h. Subconfluent cell mono-layers were washed with PBS and treated with PBS or exosomes from either H69 or CCA cells in serum- and growth factor-freed, and exosome-depleted medium for the indicated time. Cells were then washed thrice with cold PBS, fixed with 4% PFA and completely washed with PBS-Tween 20 (0.02% v/v). After blocking with 1% (w/v) BSA at room temperature for 1 h, cells were treated with anti-mouse

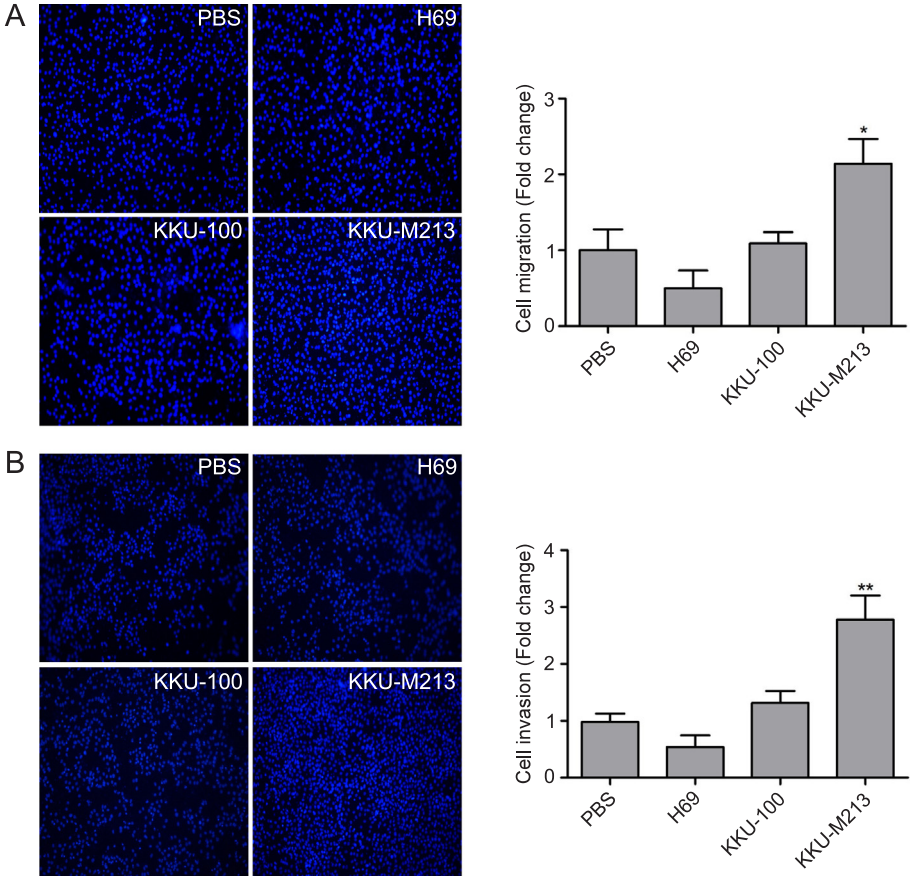

Fig. 2. Effects of H69- and CCA-derived exosomes on H69 cell migration and invasion. (A) KKU-M213-derived exosomes induced H69 cell migration. H69 cells were cultured in the upper chamber of trans-well in exosome-depleted and growth factor-freed medium. Cells were then incubated with PBS or 200 μg/ml of exosomes isolated from H69, KKU-100, and KKU-M213 cell lines for 48 h. Non-migrated cells were removed by cotton swab. Migrated cells in the lower chamber of trans-well were stained with DAPI and observed under a confocal fluorescence microscope. (B) KKU-M213-derived exosomes induced H69 cell invasion. The invasion assay was performed as in the migration assay except a thin layer of matrigel was placed on top of the insert in the upper chamber of trans-well. Non-invaded cells were removed by cotton swab and invaded cells in the lower chamber of trans-well were stained with DAPI and observed under a confocal fluorescence microscope. Bar graphs are quantitative analyses of the number of migrated and invaded cells. Data represent means ± S.E.M. (n = 5) and presented as fold change. *$p < 0.01$; and **$p < 0.001$ compared to PBS control (ANOVA).

E-cadherin and anti-rabbit β-catenin antibodies cocktail overnight at 4 °C. Cells were washed with PBS three times for 5 min, and treated with goat anti-mouse IgG (H + L) Alexa 488 and anti-rabbit IgG Alexa 568 (Invitrogen; CA, USA) in the blocking buffer at room temperature for 1 h. The cells were subsequently washed with excess volumes of PBS four times and stained with DAPI. Finally, each slide was mounted and examined by a confocal fluorescence microscopy (Fluoview fv10i, Olympus). Representative images were chosen and digitally recorded at the same sensitivity and magnification.

2.11. Statistical analysis

The statistically significant differences among groups were compared using one-way analysis of variance (ANOVA) followed by Tukey–Kramer post hoc. Data were analyzed by using the statistical software package, GraphPad Prism version 5.0.

3. Results

3.1. Characterization of exosomes released from CCA and human cholangiocyte cells

To investigate the potential role of CCA cells derived exosomes in tumor progression, the exosomes isolated from normal human cholangiocyte cells (H69) and two human CCA cell lines (KKU-M213 and KKU-100) were first analyzed by electron microscopy. As shown in Fig. 1A–C, electron microscopy of negatively stained exosome preparation showed a characteristic saucer-like structure with a diameter ranged from 40 to 100 nm and crescent shaped membrane invagination that is limited by a lipid bilayer. SDS gel followed by Western blotting of whole cell lysates and exosome fractions revealed the well-known multi-vesicular body markers flotillin-1, TSG101, tetraspanin CD81 and CD63 proteins, which were abundant in the exosomes of both H69 and CCA cells (Fig. 1D). In addition, we have determined the viability of H69, KKU-100 and KKU-M213 cell lines at the time of culture mediums were collected for exosome isolation by trypan blue staining assay. We found that more than 97% of cells were viable, indicating that exosomes are released from viable CCA and cholangiocytes cells (data not shown). The results suggest that, in CCA patients, these micro-vesicles are synthesized in cholangiocarcinoma cells and may be secreted into the circulation.

3.2. KKU-M213 exosomes promote H69 cell migration and invasion

Increases in cell migration, invasion, and proliferation are hallmarks of cancer progression. The effects of CCA cells derived exosomes on H69 cell migration, invasion, and proliferation were, therefore, examined. We cultured H69 cells on top of trans-well for cell migration assay. For invasion assay a thin layer of matrigel was layered on top of trans-well chambers. As shown in Fig. 2, exosomes derived from KKU-100 cells did not induce H69 cell migration (Fig. 2A) or invasion (Fig. 2B) compared to PBS treated set. In contrast, exosomes isolated from KKU-M213 cells not only increased the migration (Fig. 2A) but also markedly increased the invasion of H69 cells (Fig. 2B). Of note, exosomes from H69 cells failed to alter both H69 cell migration and invasion. This was not due to the effect of differential exosome uptake by H69 cells. Indeed, the exosomes isolated from all cell lines were able to either be internalized or attached onto normal human cholangiocytes (data not shown). Increases in cell migration and invasion may occur as a result of an increase in cell proliferation. We next examined whether adding exosomes purified from CCA cell lines to normal cholangiocyte cells in culture would promote cell proliferation. As shown in Fig. 3A, H69 cell proliferation was not affected upon exosome treatments up to 48 h as depicted by MTT assay. The effect of different set of exosomes on H69 cell proliferation was further confirmed by Ki67 staining, a marker protein for cell proliferation. Consistent with

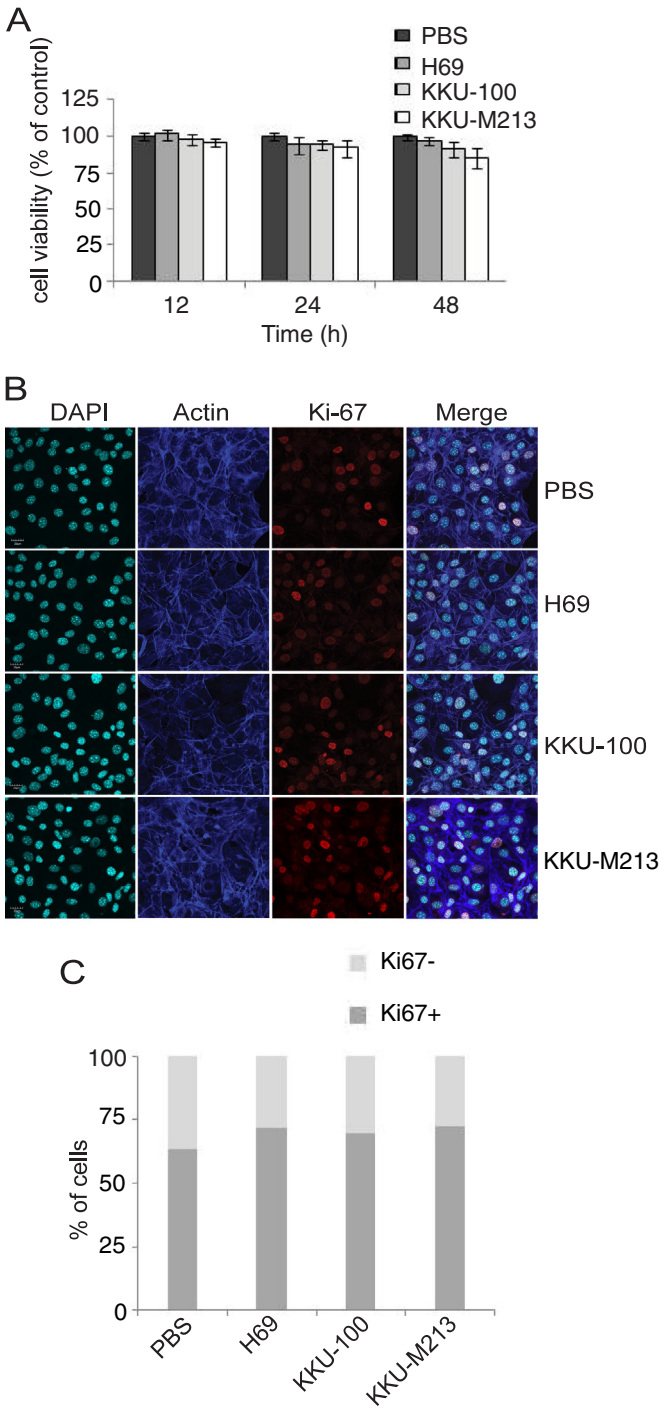

Fig. 3. Effects of exosomes on H69 cell viability and proliferation. (A) Exosomes isolated from CCA cell lines have no effect on cell viability. H69 cells were cultured in 96-well plates in complete medium. Cells were washed twice with PBS prior to incubation with PBS or 200 μg/ml of exosomes isolated from H69, KKU-100, and KKU-M213 cell lines in exosome-depleted and growth factor-freed medium for 12, 24, and 48 h. After incubation, MTT dye was added and incubated for 4 h. DMSO was added to dissolve the formazan crystals and colorimetric measurement was made at OD 590 nm. Data are means ± S.E.M. (n = 3) and presented as % of control (PBS). (B) Effect of exosomes on Ki-67 protein expression in H69 cells. H69 cells were routinely cultured on cover slips in 24-well plates for 24 h, washed twice in PBS and incubated with either PBS or 200 μg/ml exosomes isolated from H69, KKU-100, and KKU-M213 in exosome-depleted and growth factor-freed medium for 48 h. Cells were fixed by 4% PFA and stained with anti-Ki-67 antibody. Cell nuclei were stained with DAPI and visualized under a confocal microscope. (C) Bar graphs represent percentages of Ki-67 positive cells among total number of cells in randomly observed fields (n = 3).

the MTT data, the expression pattern of Ki67 protein was almost similar in all conditions upon treatment with exosomes isolated from CCA cells (Fig. 3B and 3C), suggesting that CCA-derived exosomes do not enhance or suppress normal cell proliferation.

3.3. Proteomic analysis of exosomes derived from human cholangiocyte and CCA cells

Exosomes isolated from KKU-M213 cells, but not from other set of cells tested, promote H69 cell migration and invasion, suggesting the different sets of proteins are carried by exosomes in normal and CCA cell lines. We further investigated the protein composition in exosomes isolated from KKU-M213 and H69 cells by proteomic approaches. First, we separated the total exosomal proteins (30 μg) from KKU-M213 and H69 cells by 3-8% gradient SDS-PAGE and stained with Coomassie blue. The protein bands were distinctly different between two sets of exosomes (data not shown). To identify the protein profile of CCA and H69 exosomes, individual slice of gels were subjected to in-gel trypsin digestion followed by mass spectrometry. The data analysis using Mascot database has identified proteins and their score for both H69 and KKU-M213 derived exosomes (Tables 1, S1 and S2). As shown in Table 1, a number of proteins were exclusively found in the exosomes purified from normal human cholangiocyte cells, not in KKU-M213 cells. Among the identified proteins, 10 proteins were found to be expressed in both exosome samples. Interestingly, the exosomes isolated from KKU-M213 cells accommodated 38 distinct proteins, compared to those of H69 cells, including cancer related proteins such as galectin-3-binding protein, prostaglandin F2 receptor negative regulator, 4F2heavy chain, integrin-β1, major vault protein and integrin β1 (Table 1). Our proteomic results suggest that CCA may promote cell invasion and migration via oncogenic molecules carried by exosomes.

Proteomics of shed vesicles, like exosomes, substantially contribute to the understanding of biological functions of exosomes. Ontology enrichment is the most ubiquitous type of functional analysis, which evaluates relative representation of biological functions, or ontology terms, such as pathways and cell processes, for the proteomic profile of interest. In our study, the Gene Ontology (GO) analysis by open source panther database was employed for prediction of any specific proteins or pathways that may affect H69 cells motility upon treatment with KKU-M213 exosomes. As shown in Fig. 4A and B, different classes of proteins with a variety of functions were identified in two different sets of exosomes purified from cholangiocyte and CCA cell lines. H69 exosomes contain higher chaperone and cytoskeletal proteins (Fig. 4A). While, cell adhesion molecules, extracellular matrix protein, oxidoreductase, receptor, transporter and protease were identified only in KKU-M213 exosomes (Fig. 4B), indicating that CCA derived exosomes are packed with enormous functional ability and/or potential to manipulate recipient cells in a wider aspect and that different to those of normal cholangiocyte cells. Interestingly, pathway classification revealed that only the exosomal proteins from KKU-M213 cells are related to metabolism and cancer-related signaling pathway such as glycolysis, pyruvate metabolism, gonadotropin releasing hormone receptor pathway, ubiquitin proteasome pathway and p53 pathway (Fig. 5A and B). The number of proteins related to integrin signaling pathway which plays an important role in cancer progression was increased in exosomal proteins from KKU-M213 cells compared to

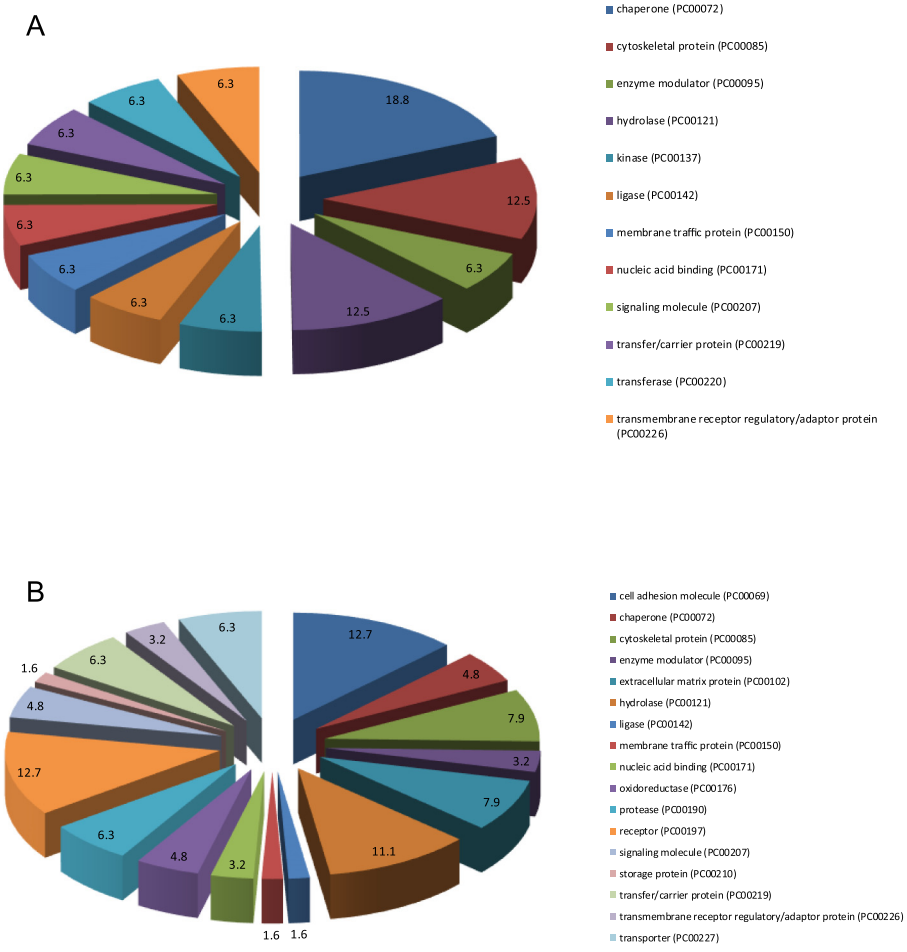

Fig. 4. Proteomic analysis of purified exosomes from H69 and KKU-M213 cell lines. Classification of proteins identified from H69 (A) and KKU-M213 (B) derived exosomes according to their protein classes using PANTHER Classification System.

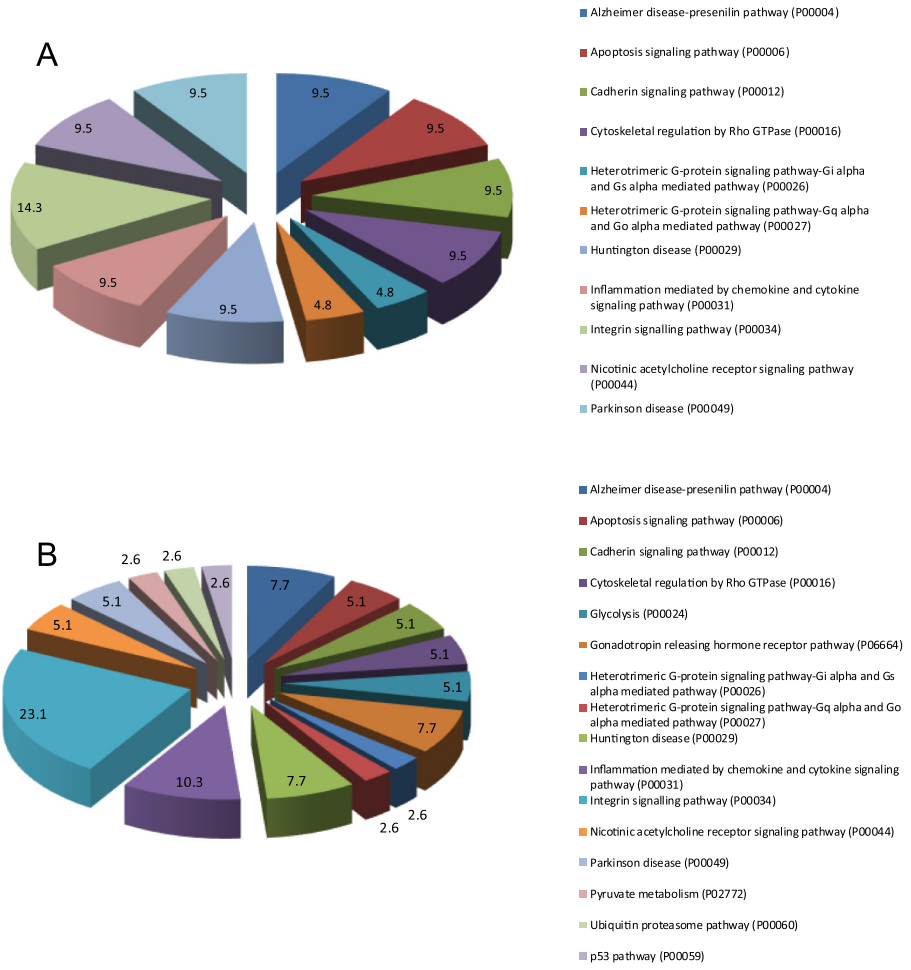

Fig. 5. Proteomic analysis of purified exosomes from H69 and KKU-M213 cell lines. Classification of proteins identified from H69 (A) and KKU-M213 (B) derived exosomes according to their related pathways using PANTHER Classification System.

normal cholangiocyte cells. Moreover, the number of proteins related to apoptosis pathway and other normal cellular activity was reduced. Taken together, the results show that proteins identified in H69 exosomes are mostly meant for maintenance of normal cellular activity compared to exosomes from KKU-M213 cells that are packed with set of proteins having significant potential to vast range of normal biological functions and disease development such as cancer.

3.4. CCA exosomes alters E-cadherin and β-catenin expression in H69 cells

Since treatment with KKU-M213 exosomes increased invasion and migration of H69 cells, we then assessed the cancer and cell motility related proteins, β-catenin and E-cadherin, by fluorescence microscopy. As shown in Fig. 6A, KKU-M213 exosomes increased β-catenin and reduced E-cadherin expression in H69 cells whereas the expression patterns remain unchanged after treatments with H69 or KKU-100 exosomes. Western blot analysis also confirmed the increase (1.7 folds) in expression of β-catenin (Fig. 6B and C). However, the phosphorylation of GSK-3β, a well-known β-catenin regulator, was not affected, suggesting that the increase in β-catenin expression in H69 cells was GSK-3β-independent. Collectively, these results indicate that exosomes from CCA cells carry cancer-specific proteins that may modulate normal cells toward tumor characteristics.

4. Discussion

Exosomes play a critical role in various pathological conditions [21]. Their biogenesis, intra-vesicular content, and transfer to recipient cells are of enormous biological interest. Having a unique molecular cargo, exosomes can reprogram the recipient cells. A number of recent studies have demonstrated that tumor derived exosomes can modulate functional geometry of recipient cells. It may help establish oncogenic niche systematically via delivery of proteins, mRNAs or miRNAs, which, in turn, promote cancer cell progression and metastasis [22]. Despite the potential importance of exosomes in the context of cancer progression, the presence of exosomes in human CCA cell line and their possible role in CCA pathogenesis have not yet been reported. Herein we demonstrated for the first time that two CCA cell lines derived from Thai cholangiocarcinoma patients and SV40-transformed human cholangiocyte H69 cells released exosomes into the extracellular media. These exosomes share similar biophysical and biochemical properties such as shape, size and specific membrane protein content of exosomes from other sources [23]. Our study provides insights into the role of CCA-derived exosomes in cancer progression. We showed that exosomes released by CCA cells *in vitro* have a potential to modulate normal cholangiocyte cell motility. Thus, exosomes released from KKU-M213 were internalized by H69 cells, deliver functional biochemical constituents and induced cell migration and invasion, but not proliferation. By means of proteomic approach, proteins identified in H69 exosomes are mostly associated with normal cellular activity. In contrast, exosomes from KKU-M213 cells are packed with a set of proteins having significant potential to intervene in cancer progression. Further, our data demonstrate that CCA-derived exosomes carried cancer-specific proteins, highlighting a novel source of diagnostic biomarkers and therapeutic target for cholangiocarcinoma.

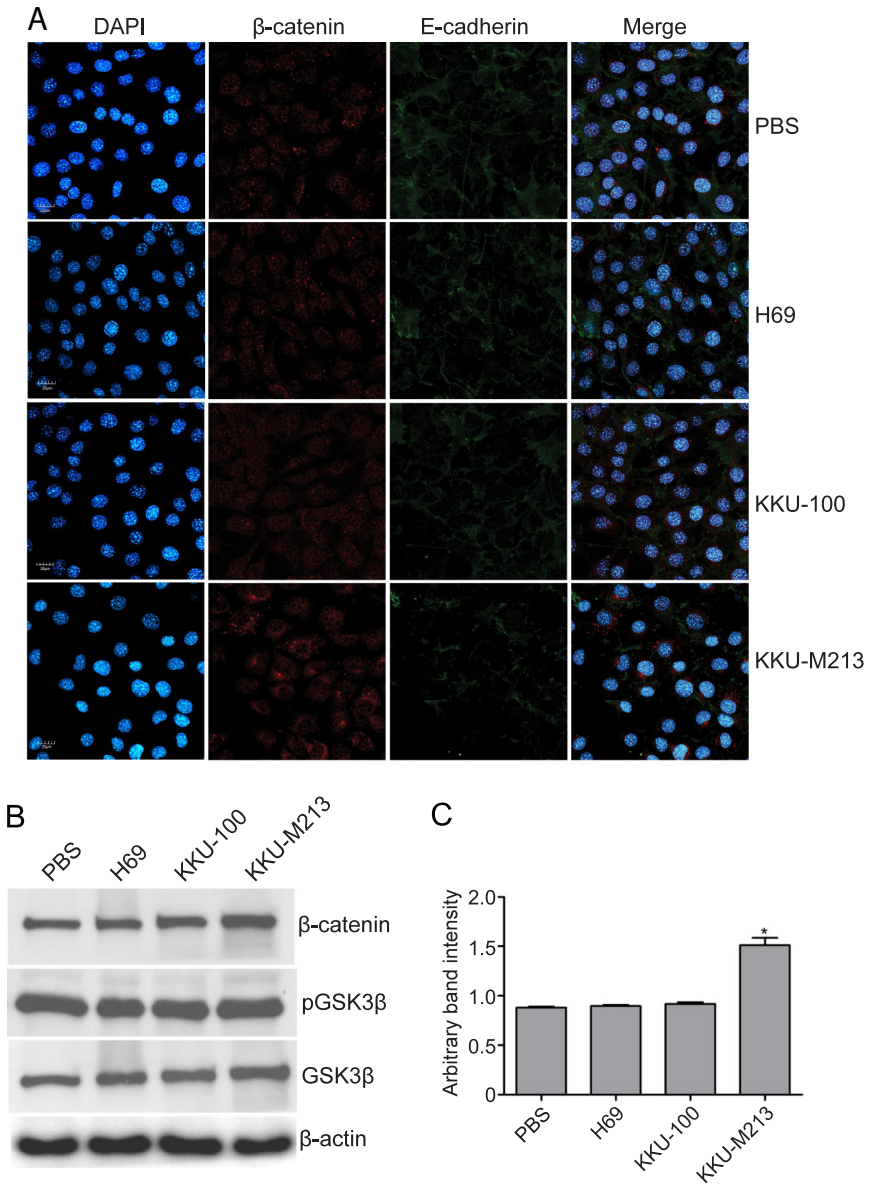

Fig. 6. KKU-M213 exosomes reduce E-cadherin and induce β-catenin expression in H69 cells. (A) Fluorescence microscopic analysis of E-cadherin and β-catenin. After treatment of H69 cells with PBS or 200 μg/ml exosomes for 24 h, cells were fixed and stained for E-cadherin and β-catenin using anti-E-cadherin (green) and anti-β-catenin antibody (red). DAPI was used as a nuclear marker (blue). Samples were visualized with a confocal laser microscope. (B) Western blot analysis of β-catenin in H69 after treatment with exosomes. H69 cells were treated with PBS or 200 μg/ml exosomes isolated from different cell lines for 24 h. Cells were then lysed and 25 μg proteins were separated by 10% SDS-PAGE and immunoblotted with respective antibodies. (C) Bar graphs representing the quantitative analysis of Western blotting of β-catenin from 3 independent experiments. Data are means ± S.E.M., $^{*}p < 0.0001$ compared to PBS control (ANOVA).

MS-based proteomics continues to contribute enormously to our understanding of the molecular composition and functions of exosomes. The concept suggests that digging out the composition and the abundance of each protein in exosomes from different sources may be useful for the identification of novel biomarker candidates, if the proteomic profiling is planned in a comparative approach [24]. The subset of proteins packed into exosomes can be functional in new environment after being transferred to other cells, which is an exhilarating new development to untie exosome saga. It has been reported that exosomes released by different cell types are different with regard to their specific cellular components [25]. Our proteomic study revealed different proteins in two sets of exosomes (Table 1). Exploring these protein profiles revealed that exosomes from H69 and KKU-M213 cells contained several proteins which are consistent with exosome biosynthesis such as transitional endoplasmic reticulum ATPase, molecular motors such as tumor susceptibility gene 101 and ubiquitin. Through our proteomic approach, we have identified exosomal proteins that are known to be associated with cancer cell adhesion, migration, and invasion. A number of these proteins have previously been identified by other researchers in similar studies. The categorization revealed some interesting facts that KKU-M213 exosomes contain broad spectrum of functional proteins involved in cancer progression more than H69 exosomes. We detected integrin α and β, lactadherin, and vitronectin in KKU-M213 exosomes. Recent studies demonstrated that integrins involved in cell growth and migration through interaction with vitronectin [26], and exogenous lactadherin directly activated AKT-dependent pathway through interaction with integrins that subsequently lead to neo-vascularization, supporting their functional roles in cancer progression.

Although the functions of exosomal proteins and lipid are well characterized, data concerning glycoprotein composition are scarce [27]. In our proteomic study, we found that the exosomes from KKU-M213

cells were enriched with a sialoglycoprotein which was identified by peptide mass fingerprinting as the galectin-3-binding protein (LGALS3BP or LG3BP). Several lines of evidence indicate the essential contribution of galectin-binding glycoproteins in different events associated with tumor growth and metastasis [28]. The role of LGALS3BP in neoplastic progression is strongly supported by numerous studies. The expression levels of this protein in sera and neoplastic tissue from cancer patients tightly correlate with poor prognosis and the occurrence of metastasis [29]. In addition, Escrevente et al. speculated that exosomal proteins may involve in exosome/target cell interactions and the LG3ALSBP protein may serve as an exosome biomarker for ovarian carcinoma [27]. Since LGALS3BP is known to bind several proteins on the cell surface such as collagens, fibronectin, galectin-3 and integrin beta1, it is possible that exosomal LGALS3BP interacts with target cells through binding with extracellular matrix proteins, which, in turn, triggers the cellular signaling pathways associated with cancer progression. In agreement with this notion, Kim et al. demonstrated that suppression of LGALS3BP decreased cell motility compared to the parental cells whereas high levels of LGALS3BP led to increased cell migration [30].

Our MS analysis also identified several other proteins in KKU-M213 exosomes that have been established to play critical roles in cancer progression. Up-regulations of LAT1 and 4F2hc that fasten formation of solid tumors were detected in several cancer cell lines including CCA cells [31,32]. Tumor associated calcium signal transducer 2 protein stimulated several human cancer growth and up-regulation of this protein was associated with poor prognosis particularly in invasive cancers [33]. High levels of vault protein expression are correlated with a poor prognosis in certain cancers with some multi-drug resistant (MDR) cancer cell lines [34]. Silencing a novel endoplasmic reticulum protein KIAA1199 discovered earlier to induce cell migration in invasive cancers caused a reduction of 75% in cell migration and 80% in cell invasion [35]. Another KKU-M213 exosome protein, CD147, was reported to play important roles in hepatocellular carcinoma invasion and metastasis [36]. Epithelial cell adhesion molecule observed in KKU-M213 exosome, is a transmembrane glycoprotein that has oncogenic potential due to its ability to aid cell migration, invasion, and metastasis [37]. An aberrant over-expression of pyruvate kinase isozymes M1/M2 (KPYM) associated with aggressive tumor features has been proposed to serve as a novel biomarker and a potential treatment target for thyroid cancer [38]. Although, KKU-100 and KKU-M213 cell lines were derived from CCA patients, however only exosomes from KKU-M213 cells induced H69 cell invasion and migration. These results might be due to the differences in their malignancy characteristics. Consistent with our finding, KKU-M213 cells have previously been reported to exhibit higher motility and invasive abilities compared to KKU-100 cells [39]. Roles of exosomes in cancer cell motility, metastasis and growth have recently received wide attention. For example, Luga et al., have recently proposed that fibroblast exosomes promoted breast cancer cell protrusive activity, motility and metastasis but not tumor growth through induction of autocrine Wnt-PCP signaling mechanism [40]. Although, some of similar cancer related molecules were also present in our proteomic study, however, Wnt signaling and PCP molecules were not detected. Therefore, at present, the roles of these exosomal proteins in CCA carcinogenesis remain unclear and required further investigation. It is now well established that accumulation of β-catenin is frequently observed in many invasive cancers. In addition, Hayashida et al. postulated that a loss of E-cadherin liberates β-catenin protein from the cadherin/catenin complexes that elicits an alternative β-catenin-mediated pathway to make cancer cells more motile and invasive [41]. Consistent with this fact, we observed an acute loss of E-cadherin expression in H69 cells upon treatment with KKU-M213 exosomes. Furthermore, an increase in β-catenin expression was observed in the same treatment indicating that KKU-M213 exosome somehow negatively influenced the cadherin/catenin complex and subsequently facilitated an increase in H69 cell motility.

5. Conclusion

In conclusion, our results suggest that the exosomes from KKU-M213, an aggressive CCA cell line, might induce human cholangiocyte cell migration and invasion by a direct cell-to-cell transfer of oncogenic proteins that influence specific intracellular mechanisms related to CCA carcinogenesis. The exosomal oncogenic proteins may serve as diagnostic/prognostic and therapeutic marker candidates which will have more immediate medical applications for CCA treatment.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2015.06.024.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Conflict of interest

The authors have no conflicts of interest to disclose

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