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Role of laminin and cognate receptors in cholangiocarcinoma cell migration

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ABSTRACT

Extensive desmoplasia in cholangiocarcinoma (CCA) is associated with tumor aggressiveness, indicating a need for further understanding of CCA cell–matrix interaction. This study demonstrated laminin as the most potent attractant for CCA cell migration and the vast elevation of its receptor integrin $\beta 4$ (ITGB4) in CCA cell lines. Besides, their high expressions in CCA tissues were correlated with lymphatic invasion and the presence of ITGB4 was also associated with short survival time. ITGB4 silencing revealed it as the receptor for laminin-induced HuCCA-1 migration, but KKU-213 utilized 37/67-kDa laminin receptor (LAMR) instead. These findings highlight the role of ITGB4 and LAMR in transducing laminin induction of CCA cell migration and the potential of ITGB4 as diagnostic and prognostic biomarkers for CCA.

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Introduction

Cholangiocarcinoma (CCA) is a highly metastatic tumor arising in biliary epithelium [1]. Although the frequency of global incidence is low, CCA has progressively increased over the past few decades with the highest prevalence (>80 per 100,000 population) being in northeastern Thailand, where it is believed to be associated with liver fluke *Opisthorchis viverrini* infection, common to the region [2]. Given its poor prognosis, late detection as well as lack of effective treatment, CCA cumulative mortality rate has continued to rise and, consequently, there is an urgent need to have a better understanding of CCA biology to develop more early diagnosis and efficacious therapeutic regimens [3].

Tumor microenvironment (TME) remodeling fosters cancer development by providing physical support and conveying biological information, which later contribute to tumor proliferation, angiogenesis, cell invasiveness and cell migration [4]. In basement membrane, laminins along with type IV collagens, fibronectin, nidogens and proteoglycans constitute the major extracellular matrix (ECM) components, which dictate cell adhesion and migration during cancer metastasis [5–7]. Laminins form a cross-shape structure through an array of heterotrimers composed of various combinations of five different α , three

different β and three different γ subunits. Each trimer interacting with a specific cell surface partner, either integrin or non-integrin receptors, latter comprising 37/67-kDa laminin receptor (LAMR), dystroglycan, Lutheran blood group glycoprotein, and syndecans [8]. Binding of laminin with its specific receptor activates multiple signaling proteins, viz. Rho family small GTPases, extracellular signal-regulated kinases (ERKs), c-Jun NH₂-terminal protein kinases/stress-activated protein kinases (JNKs/SAPKs), p38 mitogen activated protein kinase (p38 MAPK), and phosphoinositide 3-kinase (PI3K) [9].

Integrins, transmembrane adhesion heterodimer receptors composed of various α - and β - subunits, mediate not only cell-cell and cell-ECM adhesion but also regulate cell proliferation, migration and ECM organization resulting in normal cellular development or tumor formation, growth, angiogenesis and metastasis [10]. There exists at least nine laminin-binding integrins, namely, $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 4\beta 1$, $\alpha 6\beta 1$, $\alpha 7\beta 1$, $\alpha 9\beta 1$, $\alpha v\beta 3$ and $\alpha 6\beta 4$ [8,11]. Interaction of laminins with $\alpha 6\beta 4$ integrin is widely recognized to be associated with aggressive progression of several cancers, such as bladder, breast, cervical, head and neck, lung and pancreas [12,13]. Unlike the majority of actin cytoskeleton-binding integrins, $\alpha 6\beta 4$ integrin interacts with intermediate filament system, allowing stable anchorage of epithelial basal

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 Supplemental data for this article can be accessed [here](#).

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surface to ECM through the formation of adhesive protein complexes called hemidesmosomes (HDs) [10]. Mediated by epidermal growth factors or phosphorylation of $\beta 4$ cytoplasmic tail by protein kinase C, an increase in dismantled HDs is often observed in cancer metastasis as a result of the release of $\alpha 6\beta 4$ integrin attachment from HDs [10]. These consecutive phenomena permit $\alpha 6\beta 4$ integrin to promote cell malignant properties by relaying downstream signals to allow rearrangement in cell structure and alteration in expressions of genes related to cancer progression, viz. *nuclear factor of activated T-cells 1 (NFAT1)*, *NFAT5*, *S100A4*, *erythroblastic oncogene B-2 (ERBB-2)* and *ERBB-3* [10].

It is well recognized CCA develops in an ECM-rich environment, where its growth and progression are nourished [14]. Laminins, in particular laminin $\gamma 2$ (LAMC2), a component of laminin-522 and -332, are elevated in CCA cell lines and tissues, and are associated with poor prognosis and metastasis [15–19]. Overexpressed laminin $\beta 1$ (LAMB1) have also been reported in sarcomatoid CCA cells [20]; however, unlike LAMC2, its roles in CCA aggressiveness have not been elucidated. In addition, our previous work revealed that one of the CCA cell lines, HuCCA-1, possessed higher invasive ability through Matrigel than migration, indicating the role of Matrigel containing mainly laminin-111 (laminin-1) [21] in induction of cell invasiveness [22]. Several types of laminin-binding integrin monomers, such as $\alpha 2$, $\alpha 3$, $\alpha 6$, $\beta 1$ and $\beta 4$, are also overexpressed in CCA cell lines and tissues, and some are associated with CCA invasive and metastatic phenotypes [18,23–27]. For example, activation of ERK 1/2 and AKT resulting from enhanced levels of integrin $\alpha 6$ subunit induces CCA migration and invasion [26]. High expressions of $\beta 6$ and $\beta 4$ integrins detected by immunohistochemical staining of patients' tissues have enabled CCA to be distinguished from other types of liver cancer (viz. hepatocellular and cholangiolocellular carcinomas) [23]. Overexpression of LAMR has also been reported in CCA and is believed to be associated with promoting adhesion, invasiveness and metastasis [28,29]. Due to the highly desmoplastic nature of CCA, further understanding of CCA cell–matrix interaction is required. Properties of laminins, integrin $\alpha 6\beta 4$ and LAMR have been separately reported in CCA, but an understanding of the role of these receptor–ligand interactions remains unclear.

The study identified laminin as the key ECM protein promoting CCA motility and revealed the distinct types of receptor responsible for laminin-induced migrating process of two CCA cell lines. In addition, the relationship of expressions of laminin, together with its receptor, with clinical pathological characteristics and prognosis of CCA patients were determined. These findings should assist in

deepening knowledge of CCA cell–ECM interaction and provide clues for better diagnosis and prognosis of CCA.

Results

Effects of ECM proteins and Matrigel on CCA cell migration

Stroma of CCA tumor nourishes growth and progression [14]. In order to evaluate the impact of ECM proteins on CCA cell motility, haptotactic migration of two intrahepatic CCA cell lines, HuCCA-1 and K KU-213, derived from Thai patients was evaluated using a Transwell assay in response to presence of ECM proteins, collagen IV, fibronectin and laminin or Matrigel. Laminin was the most potent migrating promoter of both CCA cell lines, followed by collagen IV, fibronectin and then Matrigel (Figure 1).

Basal ITGB1 and ITGB4 mRNA expressions and protein levels in CCA cell lines

Interaction of laminins with various cell surface membrane receptors results in different intracellular signaling and phenotypic responses [9]. Among those receptors, integrin $\beta 1$ (ITGB1) and integrin $\beta 4$ (ITGB4) are well documented to be related to aggressive progression of several cancer types but this has not been investigated in CCA [8]. In order to provide further insight in the latter cancer, basal mRNA expression and protein levels of these two membrane receptors were measured by qRT-PCR and western blotting respectively in four CCA cell lines, HuCCA-1, K KU-055, K KU-100 and K KU-213, which showed mRNA and protein levels of ITGB4 are higher in HuCCA-1, K KU-213 and K KU-100 (listed in decreasing order of magnitude) compared to those of normal cholangiocyte MMNK-1 cells, while ITGB1 mRNA and protein level are not significantly different among all five cell lines (Figure 2).

Immunohistochemical staining of LAMB1, ITGB1 and ITGB4 in CCA tissues

In view of the above results and our previous finding that Matrigel, containing mainly laminin-111, induced HuCCA-1 invasiveness [21], LAMB1 and ITGB4 contents in paraffin-embedded cancerous ($n = 68$) and adjacent non-cancerous ($n = 19$) bile duct tissues of CCA patients were examined by immunohistochemical staining. Immunoreactive LAMB1 was absent in 17 (89%) adjacent normal tissues (Figure 3(a)) but was observed in cytoplasm (Figure 3(c)), basal or apical surfaces (Figure 3(e)) of 40 (59%) cancerous bile duct

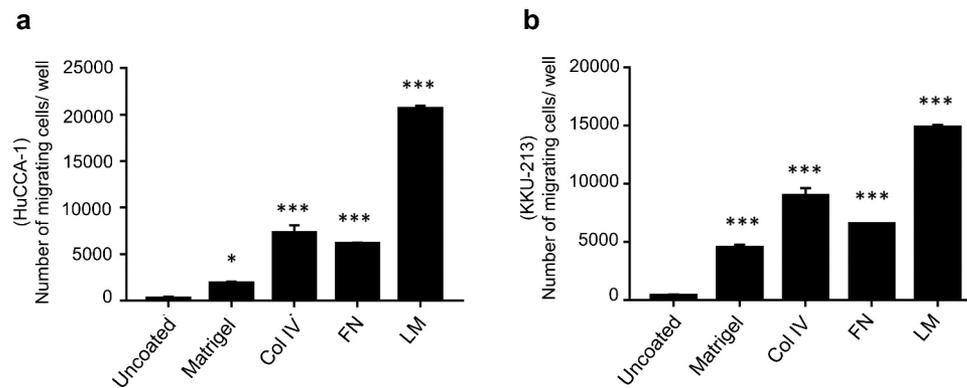


Figure 1. Effects of ECM proteins and Matrigel on haptotactic migration of CCA cell lines. Migration of (a) HuCCA-1 and (b) K KU-213 cells was determined using a Transwell system in which beneath the membrane was coated with $1.6 \mu\text{g}/\text{cm}^2$ of each individual ECM protein, including collagen IV (Col IV), fibronectin (FN), and human laminin (LM), or $1.6 \mu\text{g}/\text{cm}^2$ Matrigel. Cells (1×10^5) in $200 \mu\text{l}$ of serum-free HAM's F-12 media were seeded in the upper Transwell compartment and serum-free media were added to the lower chamber. Following six hours of incubation at 37°C , cells migrating to the membrane underside were fixed with 25% methanol, stained with 0.5% crystal violet and counted. Bar represents mean \pm SEM of three independent experiments. * p -value < 0.05 , *** p -value < 0.001 compared to uncoated control.

tissues. Presence of immunoreactive LAMB1 is significantly associated with well differentiated tissue histology (p -value = 0.031) and lymphatic invasion (p -value = 0.002) (Table 1), but not with CCA patients' survival time (Supplementary figure S1). Similar to the situation with LAMB1, immunoreactive ITGB4 was absent in all adjacent normal bile duct tissues (Figure 3(b)) but was evident in 51 (75%) cancerous bile duct tissues (in cytoplasm, cell membrane (Figure 3(d)) or basal surface (Figure 3(f))). Presence of immunoreactive ITGB4 is significantly correlated with lymphatic invasion (p -value = 0.014) (Table 1) and also with shorter CCA patients' survival time (p -value = 0.003) (Figure 3(g)). CCA patients lacking both LAMB1 and ITGB4 in bile duct have significantly longer median survival time (66 weeks, range = 27–314 weeks) compared to those with presence of LAMB1 only (median survival time = 42 weeks, range = 12–164 weeks), ITGB4 only (median survival time = 21 weeks, range = 5–358 weeks), or with both LAMB1 and ITGB4 (median survival time = 27 weeks, range = 5–264 weeks) (Figure 3(h)). As expected, immunoreactive ITGB1 showed very faint [30] staining in most cases and was comparable among cancerous and normal tissues (Supplementary figure S2).

Effects of ITGB4 knock-down on laminin-promoted CCA migration and adhesion and effect of laminin on ITGB4 distribution

Overexpressed ITGB4 was found in both CCA cell lines and patients' tissues. In order to determine if CCA

migration was stimulated by ITGB4-laminin interaction, transiently knocked-down expression of ITGB4 mRNA in two CCA cell lines (HuCCA-1 and K KU-213) shown to have high ITGB4 expression, through transfection with siITGB4 was investigated using a laminin-coated Transwell haptotactic migration assay as described above. HuCCA-1 cells with 60% loss in basal ITGB4 mRNA level exhibited $34 \pm 11\%$ decrease in motility compared to negative control siRNA transfection (Figure 4(a)), while complete loss of basal ITGB4 mRNA in K KU-213 cells had no significant effect on cell migration in a similar laminin-coated Transwell assay (Figure 4(b)), indicating role of laminin-ITGB4 interaction in promoting migratory ability of HuCCA-1 but not K KU-213.

To evaluate the ability of laminin to induce HuCCA-1 adhesion, cells were allowed to adhere on laminin for 3 hours. Compared to uncoated condition, the adhesion property of HuCCA-1 to laminin was increased by 2.0 ± 0.2 folds and silencing ITGB4 mitigated laminin-promoting adhesion by $21 \pm 1\%$ compared to siNeg-transfected control cells (Figure 4(c)). Additionally, immunofluorescence of HuCCA-1 that have been stained with anti-ITGB4 antibody and phalloidin revealed that laminin profoundly enhanced cell spreading and formation of lamellipodia at the leading edge (Figure 5: L and insert), where colocalization of ITGB4 and actin was confined in the lamella area (area inside the cell body behind lamellipodia). At the trailing, ITGB4-actin colocalization was observed mainly at proximal part but did not extend to the tip of the retraction fiber, where

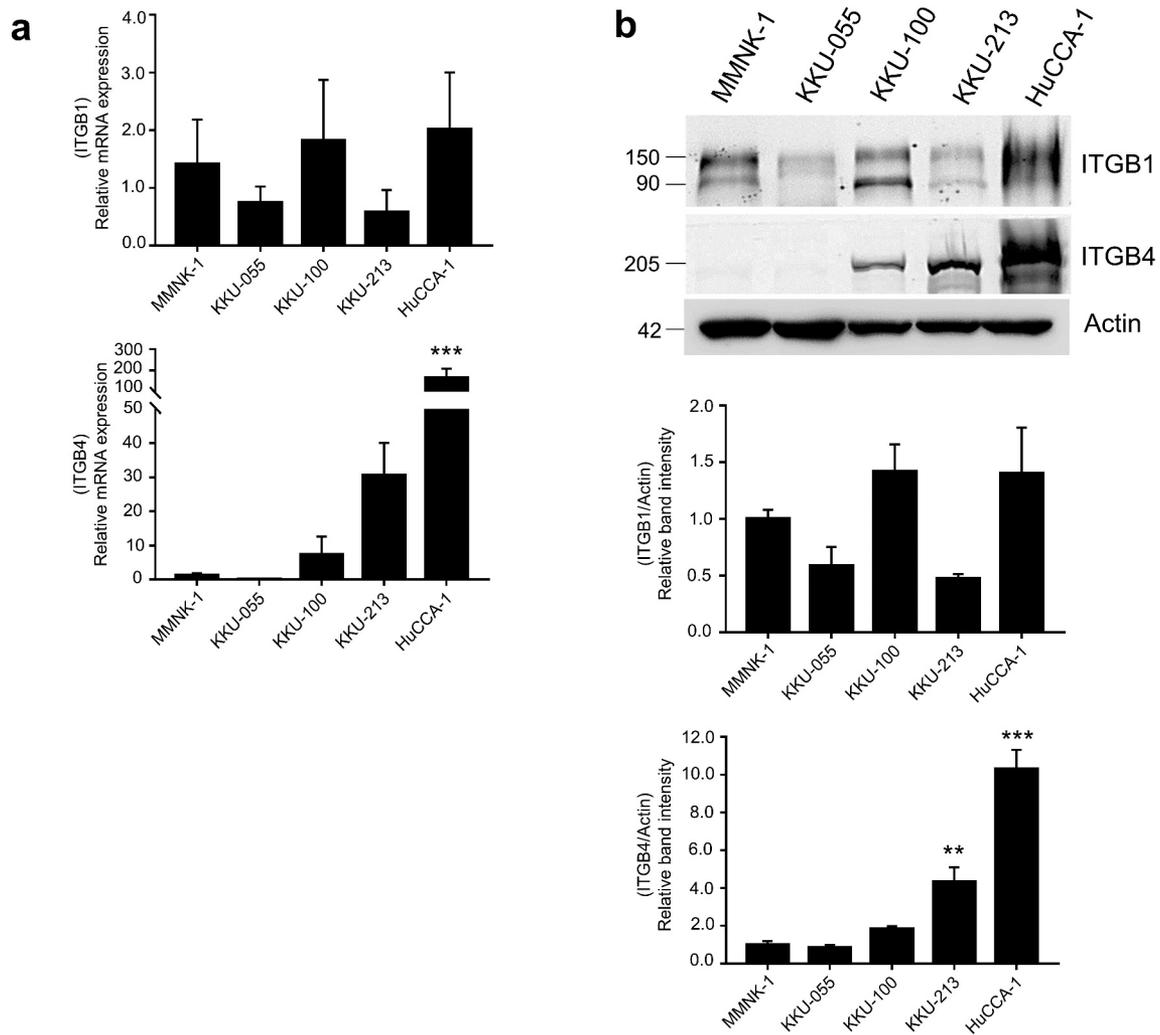


Figure 2. Basal ITGB1 and ITGB4 mRNA expressions and protein levels in CCA cell lines. (a). Relative mRNA level was measured using SYBR Green-based quantitative RT-PCR normalized to 18S rDNA and compared to that of cholangiocyte MMNK-1 cell line using $2^{-\Delta\Delta Ct}$ formula. (b). Protein level was determined by immunoblotting of cell lysates using primary specific antibodies against ITGB1, ITGB4 and actin (as gel loading control) and horseradish peroxide-conjugated secondary antibodies; immunoreactive bands were visualized using Clarity Western ECL reagent and the graphs showed band densities of ITGB1 and ITGB4 after normalization with actin in relative to MMNK-1. Bar represents mean \pm SEM of three independent experiments. ** p -value < 0.01, *** p -value < 0.001 compared to MMNK-1.

ITGB4 was mostly observed (Figure 5: arrow and insert). These data indicated the propensity of cells to migrate.

Role of LAMR in laminin-promoted KKU-213 cell migration

Other than integrins, laminin can interact with other receptors such as LAMR, which is upregulated in cancer cells [31]. Basal level of LAMR in CCA cell lines was highest in KKU-213 cells (Figure 6(a)). Transient knock-down of LAMR by siLAMR revealing $79 \pm 5\%$ reduction of LAMR mRNA expression suppressed laminin-promoted KKU-213 cell migration by $33 \pm 8\%$ compared

to the siNeg-transfected control cells (Figure 6(b)), indicating that in KKU-213 cells laminin-LAMR interaction was responsible (at least in part) for promoting cell migration. Indirect immunofluorescence for the detection of LAMR in KKU-213 cells showed that it mainly localized at the cytosol and weakly on the membrane (Figure 7).

Discussion

Prediction and treatment of CCA are notoriously difficult owing to delayed presentation of symptoms, late detection, high propensity to be unresectable as well as high rate of recurrence [2]. Developing in dense

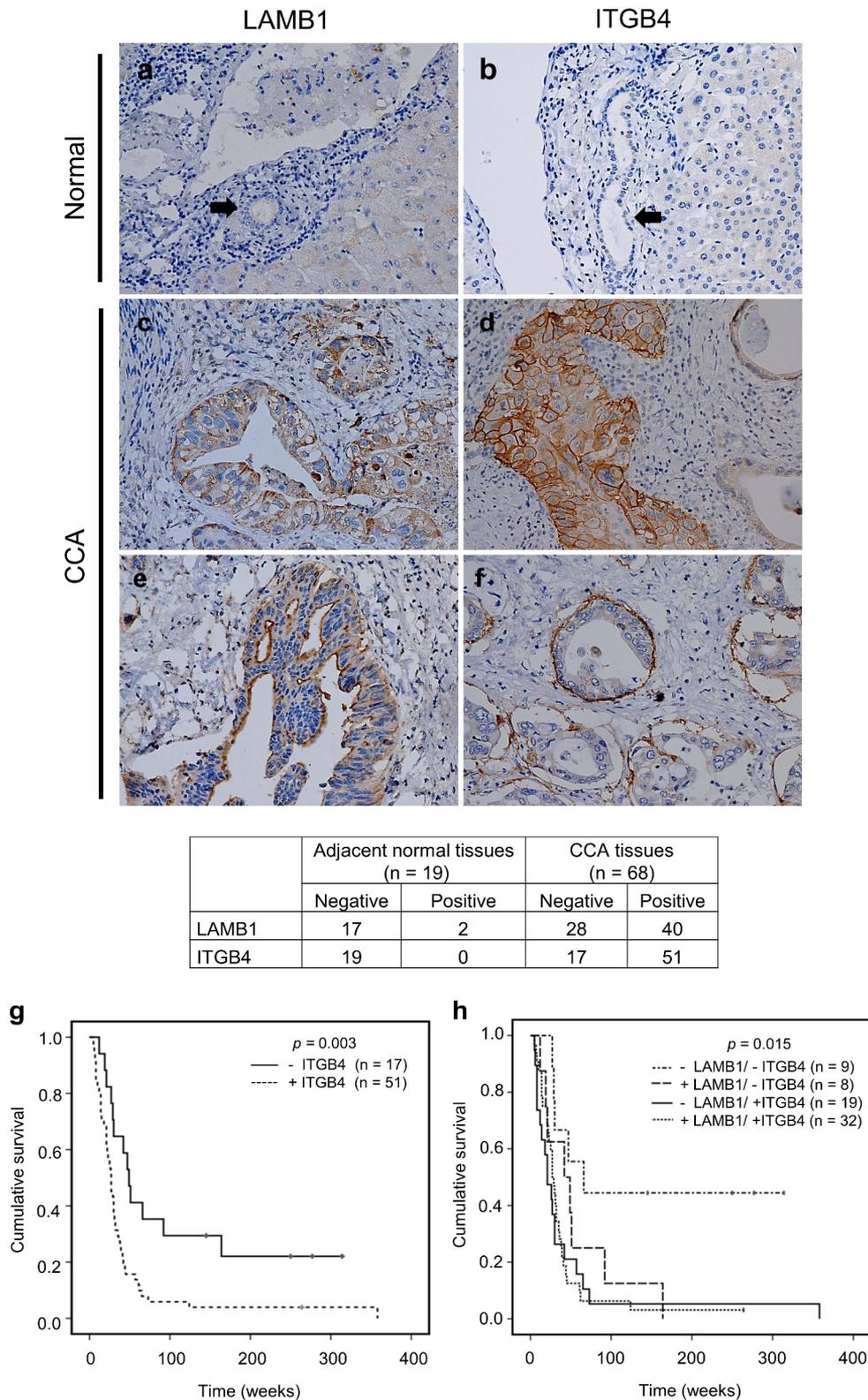


Figure 3. Immunohistochemical staining of LAMB1 and ITGB4 in bile duct tissues and survival time of CCA patients. Tumor (c–f) and adjacent normal (a, b) tissues were immunostained using anti-LAMB1 (a, c, e) and anti-ITGB4 (b, d, f) antibodies, counterstained with Mayer’s hematoxylin and visualized under a light microscope (20× magnification). Arrow indicates bile ducts. Median survival time of CCA patients depicted as Kaplan-Meier plots for presence of immunostained (g) ITGB4 and (h) LAMB1 together with ITGB4. +, present; -, absent.

Table 1. Association of clinicopathological features with LAMB1 and ITGB4 levels in bile duct tissues of cholangiocarcinoma patients at Srinagarind Hospital, Khon Kaen University, Khon Kaen, Thailand.

Variable	Presence of immunoreactive LAMB1 (n = 68)		p-value	Presence of immunoreactive ITGB4 (n = 68)		p-value
	Negative	Positive		Negative	Positive	
Age (year)						
≤ 55	14	22	0.684	11	25	0.262
> 55	14	18		6	26	
Gender						
Male	19	31	0.375	12	38	0.751
Female	9	9		5	13	
Histotype group						
Less differentiated	20	18	0.031*	10	28	0.778
Well differentiated	8	22		7	23	
Gross type						
Mass forming	23	27	0.178	13	37	0.751
Periductal	5	13		4	14	
Tumor size						
≤ 8 cm	17	29	0.307	9	37	0.134
> 8 cm	11	11		8	14	
Vascular invasion						
Absent	10	12	0.620	8	14	0.134
Present	18	28		9	37	
Lymphatic invasion						
Absent	14	6	0.002*	9	11	0.014*
Present	14	34		8	40	
Perineural invasion						
Absent	11	19	0.283	10	26	0.575
Present	17	21		7	25	
Metastasis						
Absent	13	16	0.598	9	20	0.322
Present	15	24		8	31	
Stage						
I	3	5	0.634	2	6	1.000
II	10	10		5	15	
III	15	25		10	30	

*Statistically significant.

desmoplastic stroma, CCA overproduces laminins and its cognate receptor ITGB4 but little is known regarding their involvement in regulating CCA progression [14–18,23,24]. Here, focus was placed on identifying roles of laminin and ITGB4 in CCA migration, together with the potentiality to use these two proteins as potential diagnostic and/or prognostic biomarkers of CCA.

Combined detection of multiple serum biomarkers, such as carcinoembryonic antigen (CEA), carbohydrate antigen 19–9 (CA19–9), CA125, CA242 and mucin 5AC (MUC5AC), have allowed improvements in sensitivity and specificity for early stage diagnosis of CCA [32]. However, unlike serum markers, tissue-based biomarkers are not applicable for early identification of CCA but offer diagnostic and prognostic guidance of treatment. Meta-analysis of immunohistochemical biomarkers for patients with resected CCA revealed that low expression of p27 and high expression of epidermal growth factor receptor (EGFR), fascin, MUC1 and MUC4 are associated with poor survival [33]. In the present study, both ITGB4 and laminin were detected in nearly all CCA but rarely in adjacent normal tissues, indicating ITGB4 and laminin could be potential

diagnostic biomarkers to distinguish CCA patients from normal cases. In addition, ITGB4 was a potential predictor of poor CCA prognosis as evidenced by its association with lymph node metastasis and short survival. However, there was no association of presence of laminin with patients' survival time, which may have arisen from an overriding effect of positive ITGB4 in laminin negative cases (19 out of 28 cases of negative laminin are positive for ITGB4), which most of them had short survival (Figure 3(h)). This evidence not only strengthens the possibility of using either laminin or ITGB4 detection in CCA tissues as an approach to diagnose CCA, but also suggests these two molecules as candidate biomarkers utilized in combinational detection with available CCA biomarkers to improve efficiency of diagnosis.

In the present study, all CCA cell lines used were established from Thai intrahepatic CCA patients, except for KKU-100 derived from hilar CCA, and all were associated with *O. viverrini* infection [34–36]. Among a variety of ECM proteins evaluated, laminin demonstrated the highest ability to promote migration of two patient-derived CCA cell lines, HuCCA-1 and

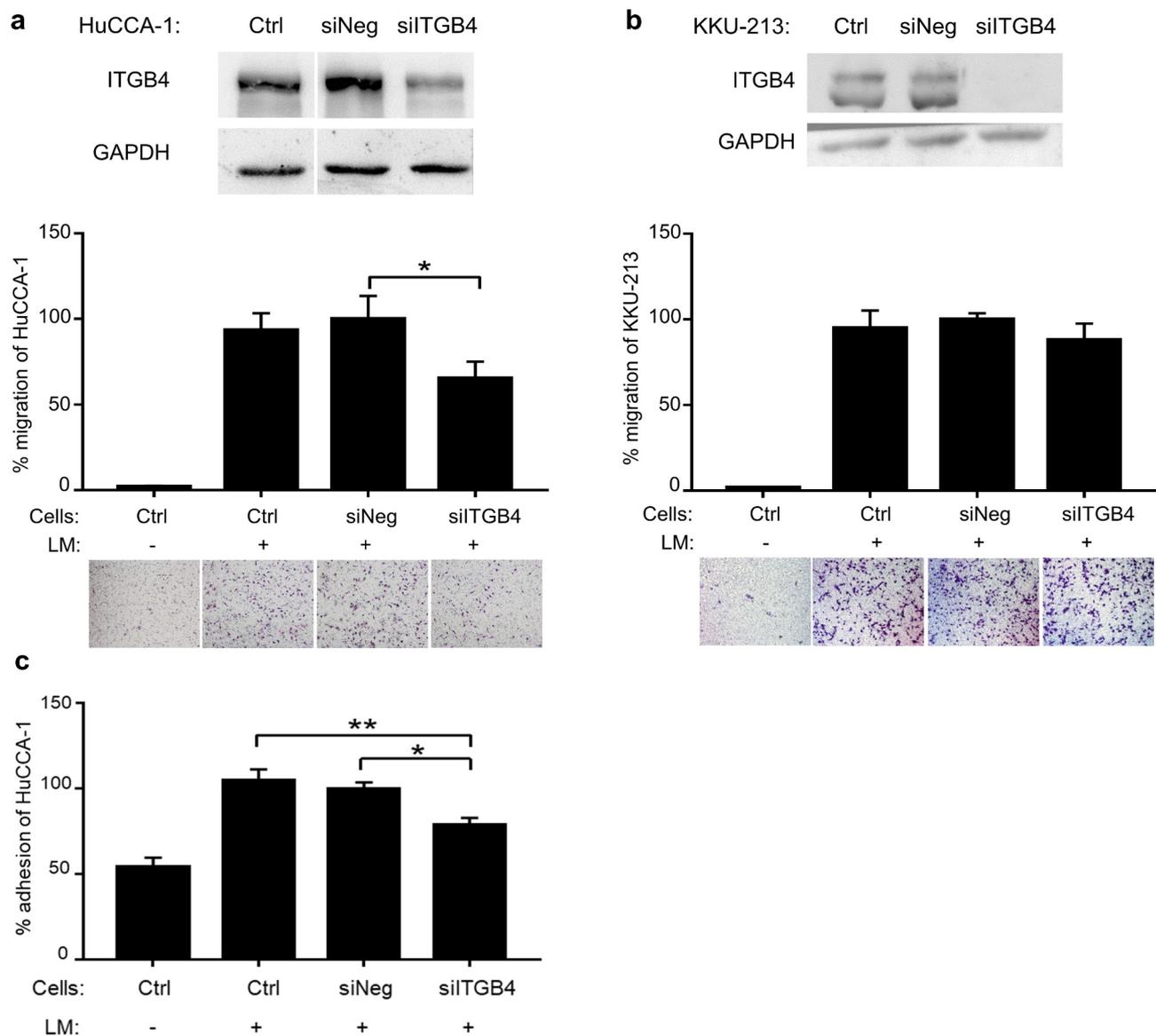


Figure 4. Effects of ITGB4 knock-down on laminin-promoted migration and adhesion of CCA cell lines. Transient reduction of ITGB4 in (a) HuCCA-1 and (b) KKKU-213 cells was performed by transfection with siRNA against ITGB4 mRNA (siITGB4) using Lipofectamin RNAiMax reagent. Transfection with nonspecific siRNA (siNeg) and non-transfected (Ctrl) cells were used as negative controls. ITGB4 level was examined by immunoblotting as described in legend to Figure 2. Haptotactic migration of cells (5×10^4) after five hours of incubation was measured using a Transwell assay as described in legend to Figure 1. (c) ITGB4-silenced HuCCA-1 cells (2×10^4) were seeded for 3 hours onto 96-well plate coated with $0.16 \mu\text{g}/\text{cm}^2$. Adherent cells were fixed, stained with 0.5% crystal violet, lysed with 1% SDS, and spectrophotometrically determined at 595 nm. Data present as mean \pm SEM percent migrating or adherent cells relative to siNeg in presence of laminin (LM+) of three independent experiments. * p -value < 0.05, ** p -value < 0.01.

KKKU-213. Unlike normal epithelial cells in which $\alpha 6\beta 4$ integrin binds to laminins and preserves the integrity of epithelial-ECM junction by forming HDs, phosphorylation of ITGB4 C-terminal endodomain in carcinoma cells leads to its release from HDs, subsequently causing reorganization of cell structure to support invasive behaviors [37]. Increase in $\alpha 6\beta 4$ integrin expression and its role in aggressive phenotypes of various carcinomas have been well documented [37]. The current work demonstrated the elevation of ITGB4 in three of

the four CCA cell lines compared to that of non-tumorigenic cholangiocyte MMNK-1 cell line and its magnitude of expression was high in HuCCA-1 and KKKU-213, respectively. Besides, we also showed that laminin stimulated HuCCA-1 migration and adhesion via ITGB4. Soejima et al reported elevated expression of ITGB4 in CCA cell lines and tissues collected at Japanese hospitals [23,24]. $\alpha 5\beta 1$ and $\alpha 6\beta 4$ integrins were reported as the major integrin receptors on CCA cell membrane owing to their high mRNA expressions

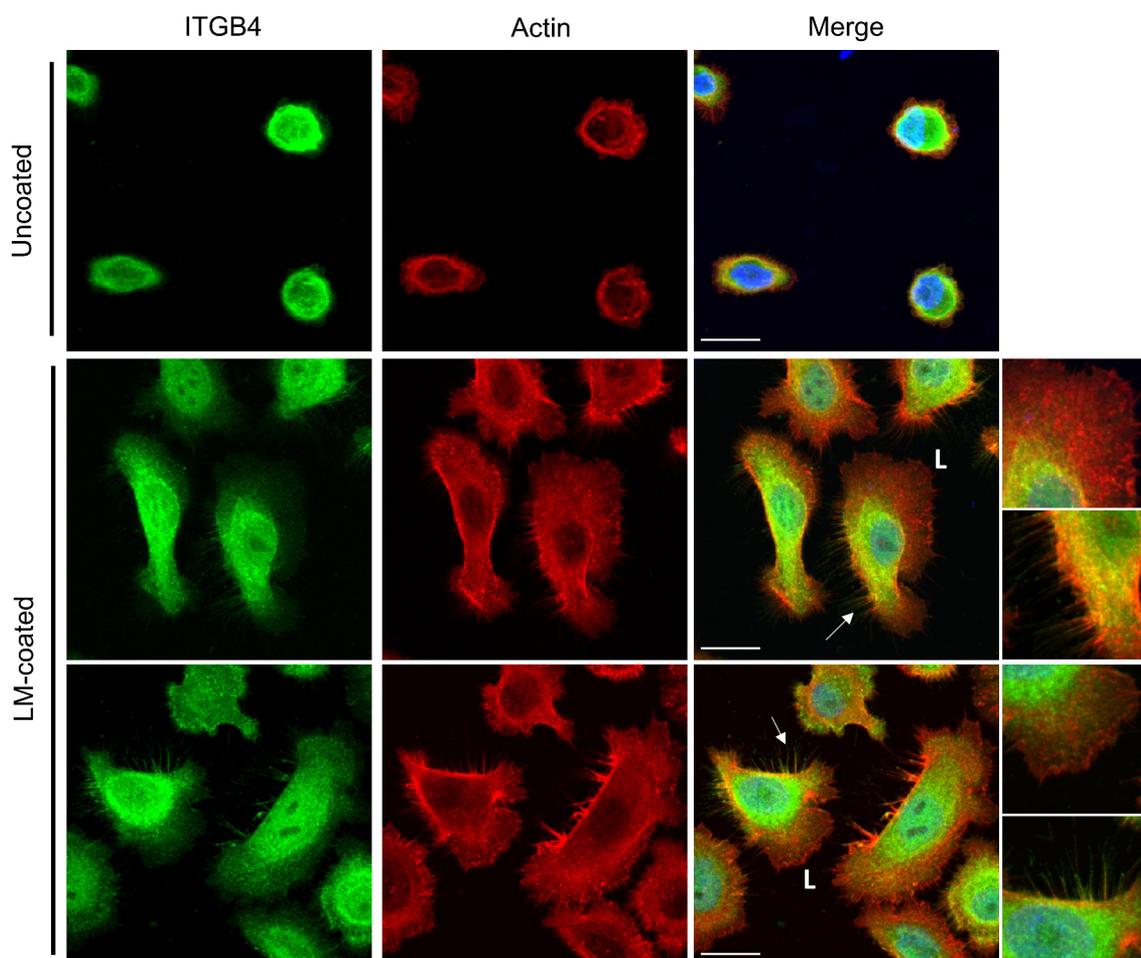


Figure 5. Effect of laminin on ITGB4 localization and actin arrangement in HuCCA-1. HuCCA-1 cells were seeded for 3 hours onto cover slip coated with $1.6 \mu\text{g}/\text{cm}^2$ laminin, fixed and stained with ITGB4 antibody, followed by Alexa Fluor 488-conjugated goat anti-rabbit (ITGB4, in green), with Alexa Fluor 546-conjugated phalloidin (actin, in red) and with DAPI (nuclei, in blue). The inserts represent enlarge areas of lamellipodia (L) and retraction fiber (white arrow). Bar, $20 \mu\text{m}$.

and their appearance on the surface of CCA cell lines shown by flow cytometry and immunofluorescence staining [27]. Interestingly, to the best of our knowledge, there has been no report of the signaling pathways in which $\alpha 6\beta 4$ integrin uses in CCA aggressiveness but one study showed that enhanced ITGA6 could activate AKT and ERK 1/2 pathways to promote CCA migration and invasiveness [26]. However, in the present study K KU-213 cell line required a non-integrin receptor LAMR, not ITGB4, for laminin-promoted migration. This phenomenon might stem from (i) K KU-213 expressing a lower level of ITGB4 compared to HuCCA-1 cell lines, (ii) over-expression of c-Met, a receptor reported to form a direct complex with $\alpha 6\beta 4$ integrin to promote HGF-dependent invasion [38], although found in both K KU-213 and HuCCA-1 cell lines, is more prominent in the latter [21], and (iii) higher LAMR expression level in K KU-213 cells compared to other CCA cell lines,

probably compensates for lower level of ITGB4. Upregulation of LAMR is observed in several other types of cancer [28,29,31]. LAMR functions in the proteolytic cleavage of laminin-111 to support ECM degradation and tumor dissemination [39], regulates tumor cell proliferation via enhanced expressions of cyclins A and B and cyclin-dependent kinases 1 and 2, and functions as a ribosomal protein crucial for protein translation [40]. In CCA, inhibition of LAMR suppresses adhesion and invasiveness of CCA cell line QBC939 [28] and LAMR promotes invasion and metastasis of CCA cell lines, RBE and QBC939, by enhancing lysyl oxidase-like-2 expression [29].

In conclusion, the study demonstrates reduction of laminin-promoted CCA cell migration in response to deficit of ITGB4 or LAMR, emphasizing the importance of CCA cell-ECM interaction in disease progression; and an association of high ITGB4 expression in CCA bile duct tissues with shorter survival time of patients, raising the

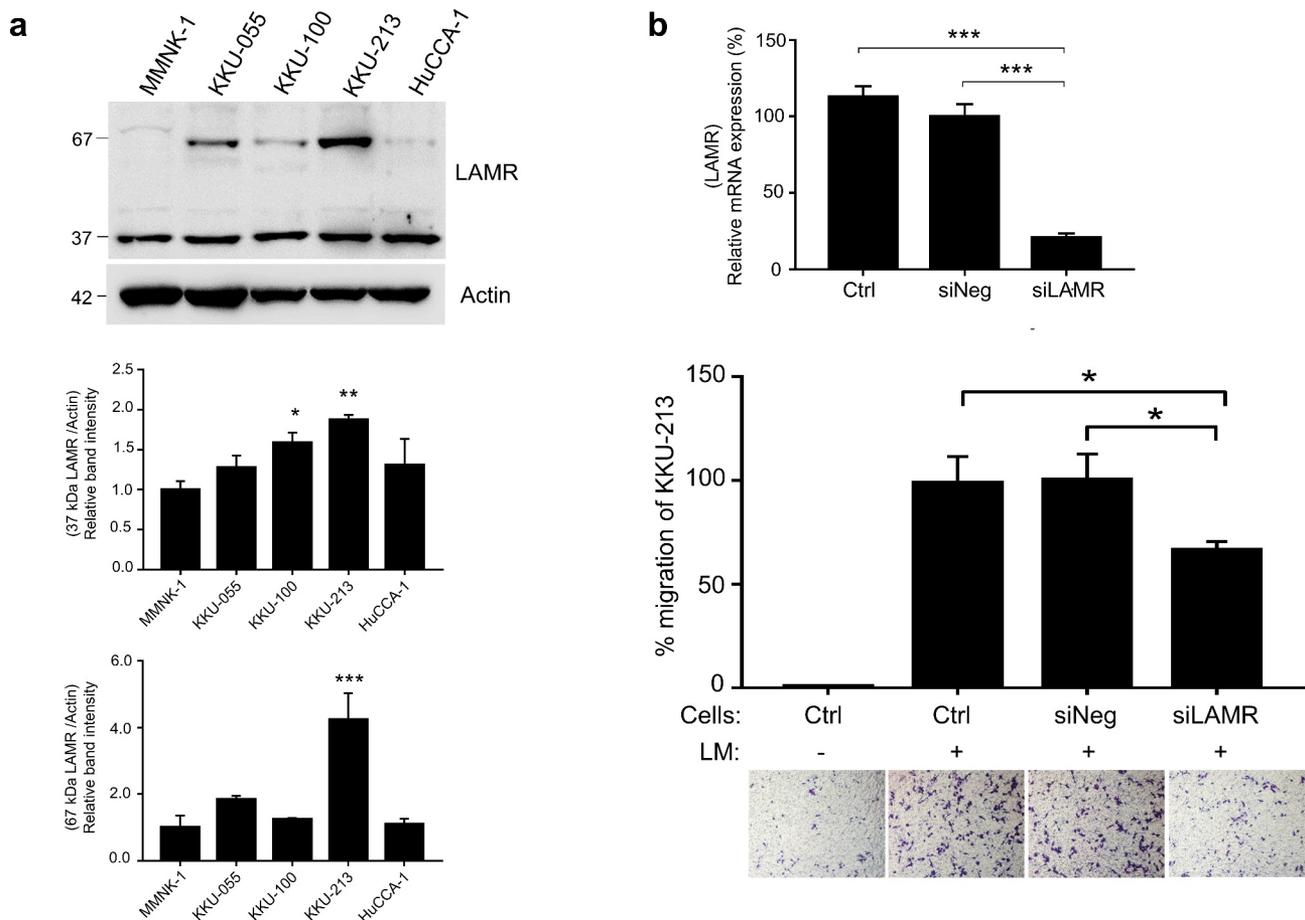


Figure 6. Basal expression of LAMR in CCA cell lines and effect of mRNA knock-down on laminin-promoted KKU-213 cell migration. (a) Basal LAMR protein level was determined by immunoblotting as described in legend to Figure 2 with actin used as gel loading control. (b) Transient reduction of LAMR mRNA level in KKU-213 cells was performed with siLAMR (upper panel) as described in legend to Figure 4. Migration of KKU-213 cells (5×10^4) were conducted in a Transwell system for five hours and results reported as mean \pm SEM percent migrating cells of three independent experiments relative to nonspecific siRNA transfected cells (siNeg) in presence of laminin (LM +). * p -value < 0.05.

possibility of employing ITGB4 combined with laminin as diagnostic biomarkers and ITGB4 alone as a biomarker of poor prognosis. The latter may guide in considering undergoing surgery or alternative strategies like cell-ECM-targeted therapy to reduce the existing high mortality rate of CCA. Thus, understanding of TME provides a platform for development of improved diagnosis and therapeutics for this highly prevalent carcinoma with high mortality that affects a sizable proportion of the rural population of northeastern Thailand.

Materials and methods

Cell culture

Thai patient-derived CCA cell lines, KKU-055 (JCRB1551) [34], KKU-100 (JCRB1568) [36], KKU-213 (previously known as KKU-M213) (JCRB1557) [41] and HuCCA-1 (JCRB1657) [35], as well as

immortalized cholangiocyte cell line MMNK-1 (JCRB1554) [42] from the Japanese Collection of Research Cell Bank (JCRB) were maintained in a humidified 5% CO₂ incubator at 37°C in HAM's F-12 media (GIBCO, Grand Island, NY, USA) containing 15 mM HEPES, 14 mM NaHCO₃, 100 U/ml penicillin G (GIBCO), 100 U/ml streptomycin (GIBCO), and 10% heat-inactivated fetal bovine serum (FBS) (GIBCO). KKU-055 and KKU-100 were developed from patients with poorly differentiated adenocarcinoma and the latter appears to be multidrug resistant cell lines [34]. Basal proliferation, migration and invasion abilities of HuCCA-1 were relatively low when compared to that of KKU-213 [21,35,43].

Small interfering RNA (siRNA) cell transfection

Thirty-six hours prior transfection with 40 nM siRNA targeting integrin β 4 (siITGB4) (sc-35678; Santa Cruz

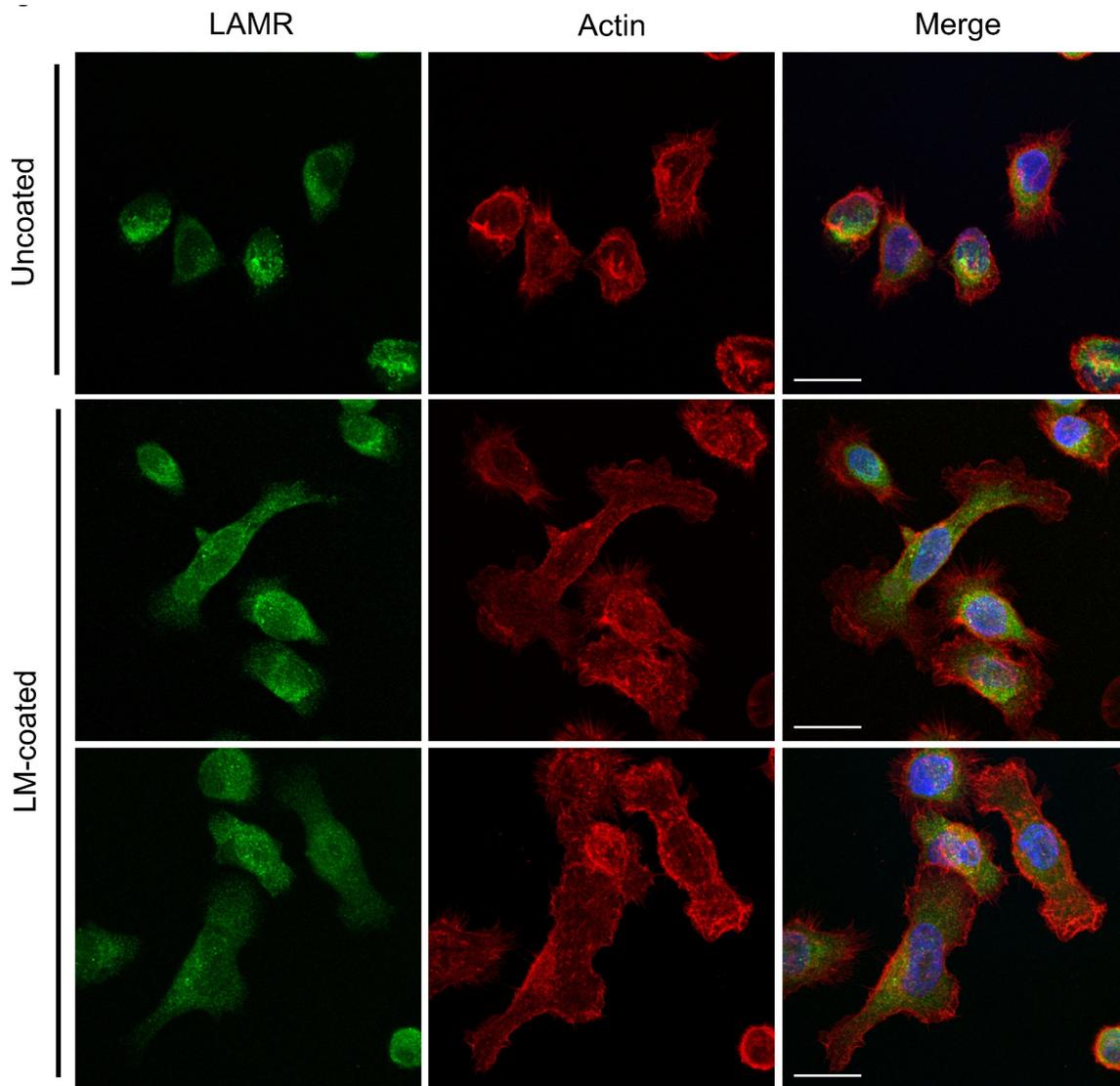


Figure 7. LAMR localization and actin arrangement of K KU-213 on laminin. K KU-213 cells were seeded for 3 hours onto 8-well slides coated with $1.6 \mu\text{g}/\text{cm}^2$ laminin, fixed and stained with LAMR antibody, followed by Alexa Fluor 488-conjugated goat anti-rabbit (LAMR, in green), with Alexa Fluor 546-conjugated phalloidin (actin, in red) and with DAPI (nuclei, in blue). Bar, $20 \mu\text{m}$.

Biotechnology, Santa Cruz, CA, USA) using Lipofectamin RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA), HuCCA-1 (2×10^5) cells were seeded in 6-well plate, while K KU-213 (2×10^5) cells were used for transfection with either 20 nM siTGB4 or 25 nM siRNA targeting 37/67 kDa laminin receptor (siLAMR) (sc-35789; Santa Cruz Biotechnology). AllStars Negative Control siRNA (siNeg) (1027280; Qiagen, Valencia, CA, USA) was employed as control. Following 24 hours of transfection, cells were incubated with fresh complete media. Migratory and adhesive abilities of cells induced by laminin were determined at 48-hour post-transfection as described below.

Haptotactic migration assay

In order to investigate the effect of ECM proteins and Matrigel on CCA cell migration, $1.6 \mu\text{g}/\text{cm}^2$ of each individual ECM protein [collagen IV (Merck Millipore, Burlington, MA, USA), fibronectin (Merck Millipore), or human laminin (Merck Millipore)] or $1.6 \mu\text{g}/\text{cm}^2$ Matrigel (Corning, Corning, NY, USA) was coated beneath an $8.0 \mu\text{m}$ pore polycarbonate membrane of a Transwell chamber (Costar, Kennebunk, ME, USA) and incubated overnight in a moist container placed in a humidified 5% CO_2 incubator at 37°C . A $200 \mu\text{l}$ aliquot of serum-free media containing 1×10^5 cells was added to the upper chamber which was then placed

into the lower compartment filled with serum-free media and container was incubated as described above for a further 6 hours. Migrating cells attached to Transwell membrane underlayer were fixed with 25% methanol, stained with 0.5% crystal violet and counted. ITGB4 and LAMR knock-down CCA cells (5×10^4 cells) were assayed using laminin-coated membrane and allowed to migrate for 5 hours.

Cell adhesion

To determine the effect of laminin on CCA cell adhesion, $0.16 \mu\text{g}/\text{cm}^2$ of laminin was coated on 96-well plate and incubated overnight in a moist container placed in a humidified 5% CO_2 incubator at 37°C . A $100 \mu\text{l}$ aliquot of ITGB4 knock-down HuCCA-1 cell (2×10^4 cells) suspension in serum-free media was seeded onto 96-well plate coated with laminin and allowed to adhere for 3 hours. Thereafter, non-adherent cells were removed by washing with phosphate-buffered saline (PBS) twice. Adherent cells were fixed with 4% paraformaldehyde for 10 minutes and stained with 0.5% crystal violet in 20% (v/v) methanol for 10 minutes. The dye was released from the cells by addition of 1% sodium dodecyl sulfate ($200 \mu\text{l}$), and the absorbent of the dye solution was measured at 595 nm.

Quantitative reverse-transcriptase (qRT)-PCR

RNA was extracted from cells (70–80% confluence) using an Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare, Munich, Germany) and determined concentration/quality with a Nano Drop UV-spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA ($1 \mu\text{g}$) was reverse-transcribed employing random hexamer primers and Improm-II™ reverse transcriptase (Promega BioSciences, Madison, WI, USA). QPCR was then carried out in a CFX96 Touch System (Bio-Rad, Hercules, CA, USA) with a $10\text{-}\mu\text{l}$ reaction mixture containing 25 ng of cDNA, 1X FastStart Universal SYBR Green Master cocktail (Roche, Bazel, Switzerland) and 5 pmol of primer pair specific for laminin receptors: ITGB1 (5'-GTGGTTGCTGGAATTGTTCTTA-3'/5'-AGTGTGTGGGATTTGCAC-3'), ITGB4 (5'-ATAGAGTCCCA GGATGGAGGA-3'/5'-GTGGTGGAGATGCTGCTGT A-3') and LAMR (5'-CTCAAGAGGACCTGG GAG-3'/5'-TGCGCAGCAGCAAACCTTCAGC-3') and 18S rDNA (5'-CCATCCAATCGGTAGTAGCG-3'/5'-GTAACCCG TTGAACCCATT-3') as internal control [44]. Thermocycling conditions were as follows: 95°C for 5 min; 40 cycles of 94°C for 45 s, 55°C for 30 s and 72°C for 30 s. Relative mRNA level was quantified using $2^{-\Delta\Delta\text{Ct}}$ method.

Western blotting

Proteins were extracted from cells (80% confluence) with lysis buffer (150 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 0.1% SDS, 1% sodium deoxycholate, 1% Nonidet P-40, 1X protease inhibitor cocktail (Roche), 50 mM NaF, 2 mM Na_3VO_4 , 40 mM β -glycerophosphate and 1 mM dithiothreitol), separated by 10% SDS-PAGE, transferred onto nitrocellulose membrane, and incubated with primary antibodies (viz. goat anti-ITGB1, rabbit anti-ITGB4 and rabbit anti-LAMR antibodies (sc-9936, sc-9090 and sc-20979, respectively; Santa Cruz Biotechnology)) (1:1000 dilution) followed by rabbit anti-goat or goat anti-rabbit horseradish peroxidase (HRP)-conjugated IgG secondary antibodies (sc-2768 and sc-2004 respectively; Santa Cruz Biotechnology). Clarity Western ECL reagent (BioRad, Hercules, CA, USA) was employed to obtain chemiluminescent signals recorded by a G-Box Chemi XL system (Syngene, Cambridge, UK). Actin or GAPDH used to normalize gel loading was detected with rabbit anti-actin (SAB5600071; Sigma-Aldrich, St. Louis, MO, USA) (1:2000) or goat anti-GAPDH (sc-48166; Santa Cruz Biotechnology) (1:2000) primary antibodies followed by the appropriate secondary antibodies as described above. The band intensity was quantified using Fiji software [45].

Immunohistochemical assay

Archived CCA (68 samples) and adjacent non-cancerous (19 samples) paraffin-embedded tissues obtained from patients who had undergone liver resection at Srinagarind Hospital, Khon Kaen University, Khon Kaen, Thailand were deparaffinized in xylene and rehydrated in a gradient series of ethanol. Antigens were retrieved under pressure for 7 min in 10 mM citrate buffer solution (pH 6.0) followed by quenching of endogenous peroxidase activity with 3% (v/v) H_2O_2 for 30 min. Following incubation with 5% normal horse serum for 1 hour, sections were separately treated with goat anti-LAMB1, rabbit anti-ITGB4 and goat anti-ITGB1 primary antibodies (sc-6018, sc-9090 and sc-9936, respectively; Santa Cruz Biotechnology) (1:200 dilution) at room temperature overnight, followed with anti-goat (Abcam, Cambridge, MA, USA) or anti-rabbit (Cell Signaling Technology, Danvers, MA, USA) HRP-conjugated secondary antibodies (1:200) for 1 hour. Specimens were then stained with diaminobenzidine (DAKO, Santa Clara, CA, USA) to develop HRP signal, counterstained with Mayer's hematoxylin and visualized under a light microscope (20 \times magnification). Positivity is defined

when >20% of positively stained cells or areas were observed, otherwise the sample is considered negative. Clinicopathological features were examined by pathologists.

Experimental protocol was approved by the Khon Kaen University Ethics Committee for Human Research (HE42075) and received exemption from Mahidol University Central Institutional Review Board (MU-CIRB 2019/098.2803).

Immunofluorescence

HuCCA-1 and KKV-213 cells (2×10^4) were seeded for 3 hours onto coverslips or 8 chamber slides (SPL Life Science, Gyeonggi-do, Korea) coated with $1.6 \mu\text{g}/\text{cm}^2$ laminin. Non-adherent cells were washed with warm serum free media and adherent cells then were fixed with 4% paraformaldehyde and 2% sucrose in PBS, and permeabilized with 0.25% Triton X-100 followed by incubation with 2% BSA. Cells were treated with rabbit anti-ITGB4 (sc-9090) (1:100) or rabbit anti-LAMR (sc-20979) (1:50) (Santa Cruz Biotechnology), followed by Alexa Fluor® 488-conjugated anti-rabbit IgG secondary antibodies (1:100) (Invitrogen). Cell nuclei were stained with DAPI (1:500) (Invitrogen), F-actin was detected by phalloidin-Alexa 546 (1:400) (Thermo Scientific). The slides were mounted with Prolong® gold antifade reagent (Invitrogen), examined under a confocal microscope Olympus FV10i-DOC (ITGB4) or FV1000 (LAMR) (Olympus Corp, Tokyo, Japan). The image analysis and processing were performed using FV10-ASW 4.2 Viewer software.

Statistical analysis

Statistical analyses were performed using SPSS software version 22.0. Data of mRNA level and percent migration are reported as mean \pm standard error of mean (SEM) of three independent experiments, and mean comparison of data from more than two independent groups were analyzed using one-way analysis of variance (one-way ANOVA) with Fisher's least significant difference (LSD) post-hoc test. Correlation between clinicopathological characteristics and LAMB1 and ITGB4 expressions was evaluated using chi-square test and survival function was analyzed using Kaplan–Meier plot. Statistically significant difference is accepted when p -value < 0.05 .

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Disclosure of potential conflicts of interest

No potential conflict of interest was reported by the author(s).

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