RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2022.049

*ScienceAsia* 48 (2022): **379–386**

# Bioinformatics and qPCR analyses of laminins' cognate receptors in cholangiocarcinoma tissues reveal the integrin *ITGB4* as a potential biomarker

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*Received 15 Dec 2021, Accepted 9 Jan 2022*
*Available online 15 Apr 2022*

**ABSTRACT**: Cholangiocarcinoma (CCA), a devastating epithelial tumor arising in bile ducts with dismal prognosis and high mortality rate, has the highest worldwide incidence in Northeastern Thailand, raising a serious health public concern to the country. Due to a desmoplastic nature of CCA, understanding CCA cell-matrix interaction is necessitated. Here, an *in silico* analysis using two RNA-sequencing (Pan-Cancer Analysis of Whole Genomes and The Cancer Genome Atlas) databases and ten microarray datasets from the Gene Expression Omnibus database, was utilized to compare the mRNA expression of 19 genes of laminins' cognate receptors between CCA and adjacent noncancerous tissues. The results were further validated in tissues of Thai patients using qPCR analysis. Bioinformatics analyses revealed that there were five common dysregulated genes in the three transcriptomic databases, in which four were upregulated genes (*ITGAV*, *ITGA2*, *ITGB1*, and *ITGB4*), and one was downregulated gene (*SDC2*). Of these differentially expressed genes, qPCR analysis confirmed the elevated mRNA expression of *ITGB4* encoding integrin β4 in 97% of the CCA cases and pinpointing the *ITGB4* as a potential diagnostic biomarker for CCA.

**KEYWORDS**: cholangiocarcinoma, integrin β4, laminins' cognate receptors, transcriptomic databases, tumor biomarkers

## INTRODUCTION

The advancement in molecular profiling techniques such as microarray and RNA sequencing (RNA-seq) has resulted in an abundance of transcriptomics data. The Cancer Genome Atlas (TCGA) [1], the Pan-Cancer Analysis of Whole Genomes (PCAWG) [2], the Genotype-Tissue Expression (GTEx) [3], and the Gene Expression Omnibus (GEO) [4] are such databases that make transcriptomic data accessible to cancer researchers and are valuable tools in understanding genetic alteration and identifying potential tumor biomarkers as well as therapeutic targets.

Cholangiocarcinoma (CCA) is a malignant carcinoma arising in the biliary tracts. CCA is frequently diagnosed in the advanced stages where surgical resection becomes impracticable, leaving palliative care as the only treatment choice [5]. Its occurrence is globally rare, but effectuates a serious public health in Thailand due to the highest worldwide incidence, especially in the northeastern region (118.5 per 100 000 population), and the high mortality rate of 14% [6]. The tumor microenvironment (TME), a complex of tumor-associated stromal cells and extracellular matrix (ECM) is a dynamic niche of cancer cells that fosters tumor progression. Interaction of cancer cells and ECM plays a vital role in several biological processes that are required for cancer progression, including proliferation, invasion, angiogenesis, and metastasis [7]. CCA is usually characterized by its prominent desmoplastic and hypovascularized stroma mediated by tumor-associated stromal cells [8]. The desmoplastic stroma of CCA, exhibited particularly in the intrahepatic and the perihilar anatomical subtypes, is so profound that it outweighs malignant cells. Therefore, the TME is critical for CCA treatment, especially for the intrahepatic type which has poor prognosis and a lack of effective remedy [9].

Due to highly desmoplastic environment of CCA, deciphering the CCA cell-matrix interaction would not only provide a deep understanding of CCA progression, but also a better therapeutic intervention of this deadly disease. We previously showed that laminins, a family of cell-adhesive glycoproteins constituting to a major proportion of ECM proteins in basement membrane, were the most potent migrating attractors for CCA cell lines [10]. Laminins relay signals via multiple signal transduction pathways involving various components, viz. Rho family small GTPases, extracellular signal-regulated kinases (ERKs), c-Jun

$NH_2$-terminal protein kinases/stress-activated protein kinases (JNKs/SAPKs), p38 mitogen activated protein kinase (p38 MAPK), and phosphoinositide 3-kinase (PI3K) [11]. Cellular receptors of laminins are categorized into two groups: integrins and nonintegrin receptors; the latter are composed of basal cell adhesion molecule (*BCAM*), dystroglycan (*DAG1*), 37/67 kDa laminin receptor (*RPSA*), and syndecans (*SDC1*, *SDC2*, *SDC3*, and *SDC4*). At least nine integrins that interact with laminins – namely α1β1, α2β1, α3β1, α4β1, α6β1, α7β1, α9β1, αvβ3, and α6β4 have been identified [12]. Interaction of laminins with different cellular receptors signifies specific cell signaling and responses. Following the previous finding, integrin β4 was shown to be strongly expressed in CCA with correlation to lymphatic invasion and patients' survival. Moreover, integrin β4 was also responsible for laminin-promoted migration of integrin β4-overexpressing CCA cell lines [10]. However, the expression of other laminins' cognate receptors in CCA has not been well addressed yet.

The aim of this study is to investigate the baseline mRNA expression of laminins' cognate receptor genes in CCA tissues compared with adjacent noncancerous tissues using publicly available RNA-seq and microarray data. Moreover, qPCR analysis of CCA tissues was performed to validate the bioinformatics analysis's results.

## MATERIALS AND METHODS

### mRNA expression profile from the PCAWG (Pan-Cancer Analysis of Whole Genomes) database

The deposited RNA-seq data from the PCAWG (https://docs.icgc.org/pcawg) were retrieved to compare the expression of laminins' cognate receptors (19 genes) across tissues of CCA ($n = 18$), noncancerous adjacent to CCA ($n = 16$), hepatocarcinoma (HCC) ($n = 100$), noncancerous adjacent to HCC ($n = 53$), and normal liver ($n = 35$) from the Genotype-Tissue Expression (GTEx) [2, 3]. Expression level of 0.5 transcripts per million (TPM) was set as cutoff value. Heatmap was generated by an online software Morpheus (https://software.broadinstitute.org/morpheus). Comparison of the mRNA expression between CCA and noncancerous adjacent tissues was analyzed by the Welch's $t$-test.

### The GEPIA2 (Gene Expression Profiling Analysis) of TCGA-CCA cohort

The GEPIA2 (http://gepia2.cancer-pku.cn), a user-friendly bioinformatics web based tool, provides differential expression analysis of various kinds of cancer based on the data from the TCGA and the GTEx [1, 13]. The TCGA-CCA (denominated by the project as TCGA-CHOL) data containing 36 CCA tissues and 9 noncancerous adjacent tissues were used to compare the expression of 19 genes of laminins' cognate receptors. An absolute $Log_2$ FC (fold change) value of 1 and the adjusted $p$-value of 0.01 were set as the cutoff criteria. One-way ANOVA was used to analyze differential gene expression; genes with higher $|Log_2 FC|$ values and lower $p$ values than pre-set thresholds were considered differentially expressed genes.

### Profiling of genes in microarray database of the Gene Expression Omnibus (GEO)

The GEO (https://www.ncbi.nlm.nih.gov/geo) is a communal functional genomic database, which includes a large number of array and sequencing-based data [4]. Ten datasets of microarray expression profiles of CCA and noncancerous adjacent tissues, previously collated from the GEO database for the other bioinformatics analysis [14], were repurposed for investigating expression of laminins' cognate receptors in CCA. In brief, gene expression profiles of CCA were retrieved from the GEO using the search queries "Cholangiocarcinoma" and "human". Results were filtered to select only tissue samples with baseline expression profiling. As a result, expression data containing a total of 704 CCA samples and 165 adjacent noncancerous samples were acquired from 10 microarray datasets – namely GSE132305 [15], GSE2263 [16], GSE26566 [17], GSE32225 [18], GSE32879 [19], GSE35306 [20], GSE57555 [21], GSE66255 [22], GSE76297 [23] and GSE89749 [24]. Each experimental dataset was individually quantile-normalized, annotated, and merged with gene IDs. To minimize the artifactual variances between data from different microarray platforms, the combined datasets were again quantile-normalized and undergone $Log_2(x+1)$ transformation, where "$x$" is signal intensity. The processed data was filtered using the genes of laminins' cognate receptors ($n = 19$) obtained from the HUGO Gene Nomenclature Committee (HGNC) and only genes being present across 10 datasets were integrated and analyzed, while those absent in one or more datasets were excluded. Data processing was performed using 'R' version 4.0.2. Mean comparison between CCA tumor and noncancerous adjacent tissues was analyzed by Welch's $t$-test.

### CCA and noncancerous tissues of Thai CCA patients

A total of 30 frozen CCA and 30 matched adjacent noncancerous tissues were obtained from Thai patients with pathologically confirmed CCA, who had undergone liver resection at the Srinagarind Hospital, Khon Kaen University, Thailand. The average age of the patients was $58 \pm 10$ years with the average survival time of $1.4 \pm 1.2$ years. Experimental protocol was approved by the Khon Kaen University Ethics Committee for Human Research (HE571283) and received exemption from the Mahidol University Central Institutional Review Board (MU-CIRB 2019/098.2803).

### Quantitative (q) RT-PCR

RNA from liver tissues was harvested using TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. In brief, liver tissues were homogenized in TRIzol™ Reagent and RNA was extracted with phenol/ chloroform solution, precipitated with isopropanol, and dissolved in RNase-free water. Total RNA (1 μg) was reversely transcribed to cDNA using 0.5 μg of random hexamer, 0.5 mM dNTP, with 10 U RNase inhibitor, and 160 U ImProm-II™ reverse transcriptase (Promega, WI, USA). The system condition for real-time PCR was in a 10-μl reaction mixture containing 25 ng cDNA, 1X FastStart Universal SYBR Green Master cocktail (Roche, Mannheim, Germany), and 5 pmol of each specific primer. The primers sequences were as follows: *ITGB1* (5′-GTGGTTGCTGGAATTGTTCTTA-3′, 5′-AGTGTTGTGGGATTTGCAC-3′), *ITGB4* (5′-ATA GAGTCCCAGGATGGAGGA-3′, 5′-GTGGTGGAGATGCT GCTGTA-3′), and *GAPDH* (5′-CACCAGGGCTGCTTT TAACTCTGGTA-3′, 5′-CCTTGACGGTGCCATGGAATT TGC-3′). The PCR conditions were 95 °C for 5 min, followed by 40 cycles at 94 °C for 45 s, 55 °C for 30 s, and 72 °C for 30 s. Changes in gene expression between CCA and matched adjacent non-tumor tissues were quantified relative to the endogenous control, *GAPDH*, using $2^{-dCt}$ method, where dCt is the difference in Ct values between *GAPDH* and gene of interest. Relative mRNA levels were analysed using $2^{-ddCt}$ method, which defines upregulation as $\geqslant$ 2-fold change; downregulation as $\leqslant$ 0.5-fold change; and unchanged when fold change ranging between 0.5 and 2. Wilcoxon matched-pairs signed rank test was performed to compare gene expression.

### Statistical analysis

Statistical analyses were conducted using Graphpad Prism software version 7 (GraphPad Software, Inc., San Diego, California). Mean comparison of two independent samples was determined by the Welch's *t*-test and mean comparison of matched samples was tested by Wilcoxon matched-pairs signed rank. Genes were considered differentially expressed when $p$ value was $< 0.01$. $^{*}p < 0.01$; $^{**}p < 0.001$; $^{***}p < 0.0001$.

## RESULTS

### Expression of laminins' cognate receptors using RNA-seq data from PCAWG and TCGA

Interaction of laminins with cellular receptors denotes the specificity of cell signaling and responses. Basal mRNA expressions of 19 genes of laminins' cognate receptors were evaluated from RNA-seq data retrieved from PCAWG and TCGA. From the PCAWG, the expression of laminins' receptors across tissues of liver carcinomas [HCC ($n = 100$) and CCA ($n = 18$)], adjacent noncancerous tissues of HCC ($n = 53$) and CCA ($n = 16$), and normal liver ($n = 35$) showed the distinguishable upregulation of *ITGA2*, *ITGA3*, and *ITGB4* in CCA compared with the noncancerous adjacent tissue, the HCC tissue, and the normal liver tissue (Fig. 1A). Additionally, seven laminins' cognate receptors, including *RPSA*, *ITGAV*, *ITGA2*, *ITGA3*, *ITGA6*, *ITGB1*, and *ITGB4* were significantly overexpressed in CCA compared with noncancerous adjacent partners. In contrast, *SDC2* mRNA was downregulated (Fig. 1B).

The TCGA-CCA cohort, consisting of 36 CCA tissues and 9 adjacent noncancerous tissues, was analysed using the GEPIA2 platform. All parameters were set to default values, and genes with higher $|\mathrm{Log_2\,FC}|$ values than the pre-set criteria were considered differentially expressed genes. The GEPIA2 analysis also revealed the upregulation of 10 genes, seven of which were the same as those found in the PCAWG, while overexpression of *BCAM*, *DAG1*, and *SDC3* was solely found in the TCGA. Similar to PCAWG, *SDC2* was also downregulated in this cohort. (Fig. 2).

### Differentially expressed genes of laminins' cognate receptors in the integrated analysis of microarray datasets

To confirm the reliability of the analyzed results, the differentially expressed laminins' cognate receptors in CCA tissues from different databases were further examined. The ten individual microarray datasets consisting of 704 CCA tumors and 165 normal tissues were collated from the GEO database as detailed previously [14]. Ten out of the 19 genes – namely *DAG1*, *SDC1*, *SDC3*, *SDC4*, *ITGAV*, *ITGA2*, *ITGA4*, *ITGB1*, *ITGB3*, and *ITGB4* were expressed in significantly higher level in the CCA tissues when compared with the normal tissues. However, the mRNAs of *BCAM*, *SDC2*, and *ITGA3* were expressed in significantly lower levels in the CCA tissues compared with the normal tissues (Fig. 3).

### Validation of *ITGB1* and *ITGB4* expression in CCA tissues of Thai patients using qPCR analysis

Venn diagram depicted five common differentially expressed genes in PCGAW, TCGA and GEO datasets, of which four were upregulated genes (*ITGAV*, *ITGA2*, *ITGB1*, and *ITGB4*) and one was downregulated gene (*SDC2*) (Fig. 4). Among these upregulated integrins, the two β subunits have been well documented to be relevant to aggressive progression of several cancers; yet they have not been widely investigated in CCA. Hence, we evaluated the mRNA expression of *ITGB1* and *ITGB4* using qRT-PCR of 30 pairs of CCA tissues and their matched adjacent noncancerous tissues obtaining from Thai patients. Relative expression levels between the CCA tissues and the noncancerous counterpart of *ITGB1* was unchanged (fold change between 0.5 and 2) in the majority of cases (47%), while *ITGB4* mRNA level was increased by 136-fold

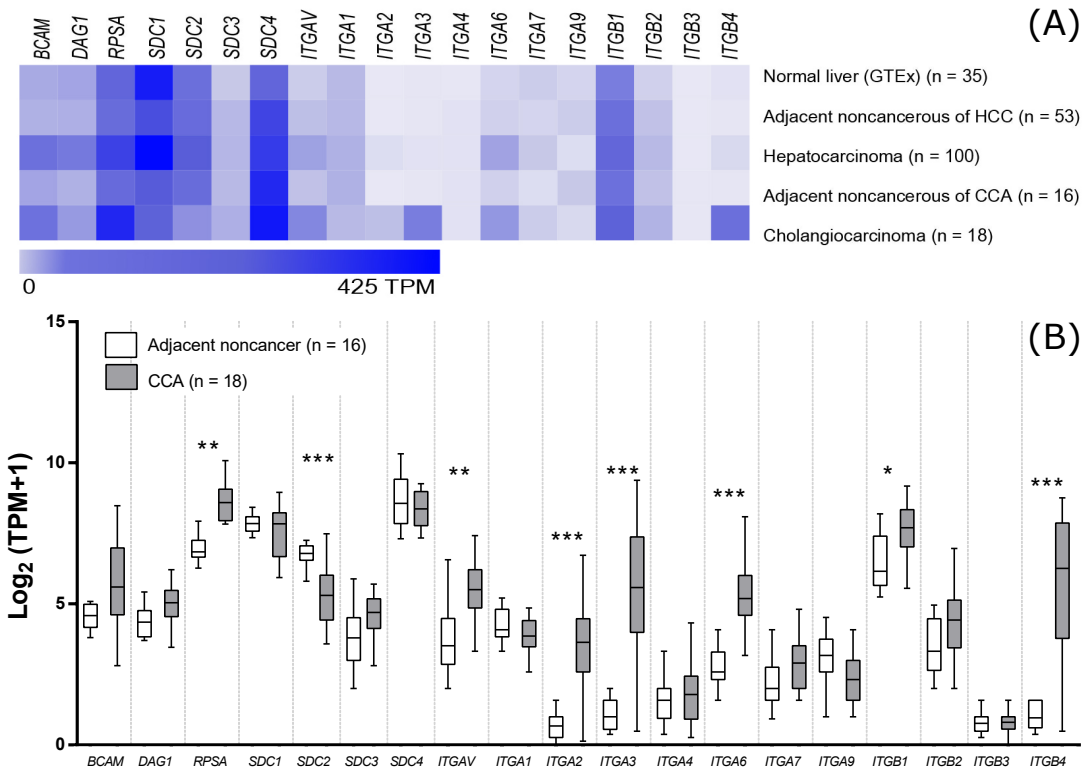


**Fig. 1** mRNA expression of laminins' cognate receptors in normal and liver cancer tissues using RNA-seq data from PCAWG and GTEx. (A) Heatmap depicting expression of laminins' receptors across tissues of CCA, HCC, their adjacent noncancerous partners and normal liver (PCAWG and GTEx databases). The heatmap generated using Morpheus software represents the median of expression level above 0.5 TPM. (B) Expression levels of mRNA of laminins' cognate receptors in patient CCA tissues (grey) and noncancerous counterparts (white) retrieved from the PCAWG. The Welch's $t$-test was applied to test for the differential expression between tumor and adjacent noncancerous tissues. $^{*}p < 0.01$; $^{**}p < 0.001$; $^{***}p < 0.0001$.

and upregulated ($\geqslant$ 2-fold change) in 29 out of 30 cases (97%) of CCA patients ranging from 4- to 2595-fold increase (Fig. 5A and 5B). Correlations between the expression levels of *ITGB1* with the survival time and the clinicopathological parameters of the disease were not statistically significant (Fig. S1 and Table S1), while those of *ITGB4* were unable to determined due to limited sample size of unchanged group ($n = 1$).

## DISCUSSION

Remodeling of TME during cancer development not only fosters cancer progression via providing physical support and transmitting biological information which are crucial for tumor proliferation, angiogenesis, migration, and invasion, but also impedes radiotherapeutic and chemotherapeutic penetrations into the stiffened stroma [25]. Understanding the impact of desmoplastic microenvironment in CCA will not only provide a deep knowledge in CCA progression, but also offer a better therapeutic intervention and prevention of reoccurrence. Various RNA-sequencing and microarray databases are publicly available to explore differentially expressed genes involved in carcinogenesis and progression, opening the door for gaining valuable information for clinical applications. Laminin has recently been demonstrated to be the most potent inducer for CCA migration [10]. In the present study, an *in silico* analysis using two RNA sequencing (PCAWG, TCGA) databases and 10 microarray datasets (GEO database) was utilized to compare the expression of laminins' cognate receptors between CCA and adjacent noncancerous tissues. The analyses revealed four common upregulated genes and one common downregulated gene. Of these, the upregulation of *ITGB4* was confirmed (using qPCR analysis) to be a potential diagnostic biomarker for CCA.

The RNA-seq data of the PCAWG project were retrieved and compared by Welch's $t$-test, and the data of the TCGA-CCA cohort were examined by the GEPIA2. From the analyses for expression of 19 genes of laminins' cognate receptors of these two databases, a total of eight common differentially expressed genes

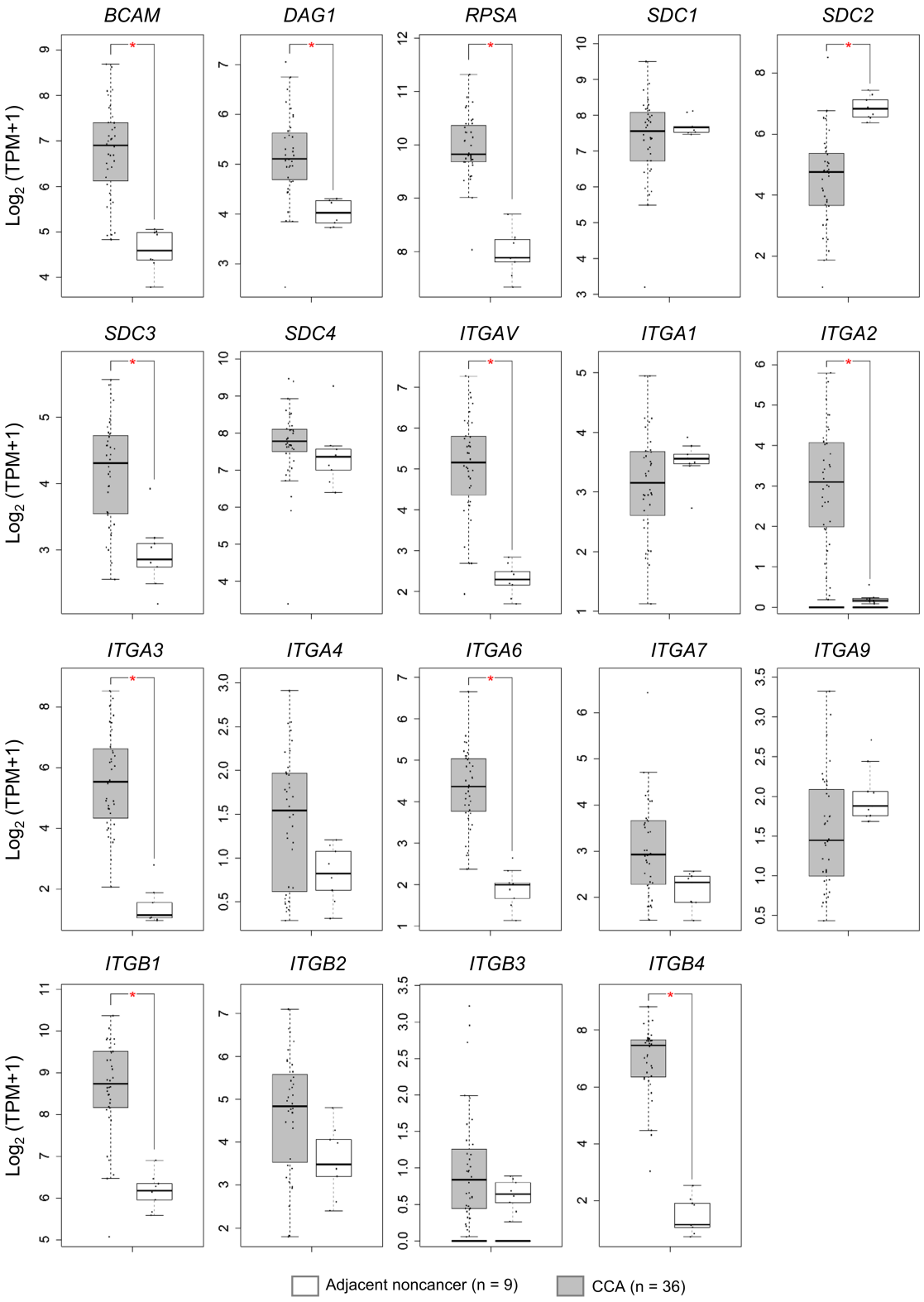


**Fig. 2** mRNA expression of laminins' cognate receptors in TCGA-CCA cohorts according to the GEPIA2 analysis. Box plot analyses compared the expression levels of the 19 specified genes in the patients' CCA tissues (grey) and adjacent noncancerous tissues (white). The $|\mathrm{Log}_2\,\mathrm{FC}|$ cutoff of 1 and a $p$-value cutoff of 0.01 were used. The method for differential analysis between the CCA and the adjacent noncancerous tissues was one-way ANOVA; $^{*}p < 0.01$.

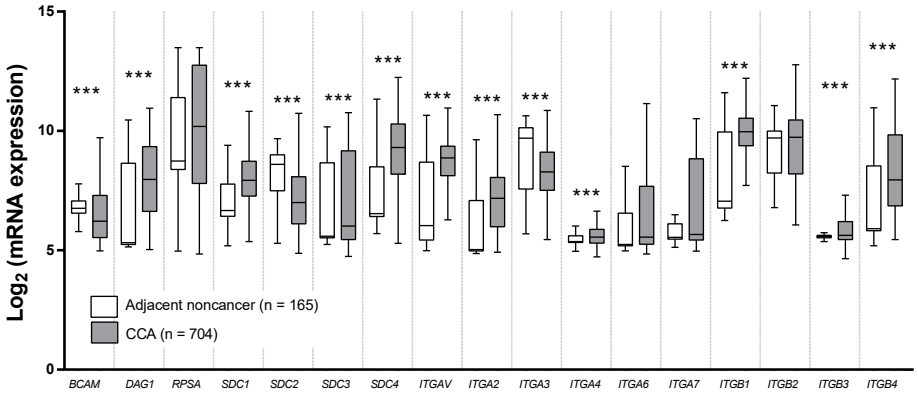


**Fig. 3** mRNA expression of laminins' cognate receptors in the CCA tissues compared with the adjacent noncancerous tissues from collated microarray datasets. mRNA expression of laminins' cognate receptors were compared between the CCA ($n = 704$) (grey) and the adjacent noncancerous ($n = 165$) (white) tissues from previously collated data [14]. Welch's $t$-testing was performed to confirm the statistical significance; $^{***}p < 0.001$.

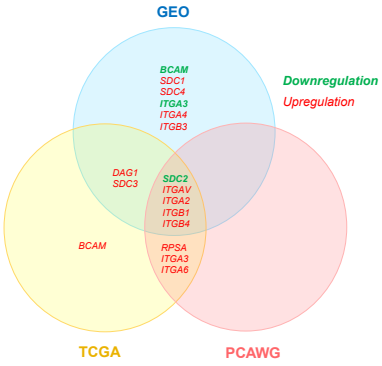


**Fig. 4** Venn diagrams illustrating the common upregulated and downregulated differentially expressed genes shared by PCAWG, TGCA and GEO databases of the CCA tissues.

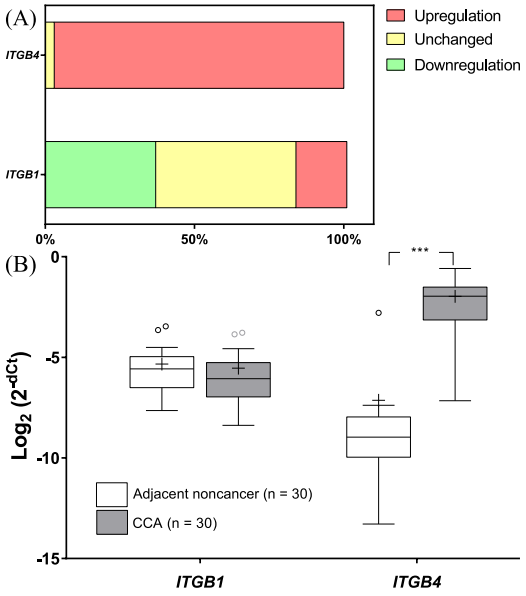


**Fig. 5** Comparative gene expression of laminins' cognate receptors in liver tissues of Thai patients with CCA compared with their matched noncancerous adjacent tissues. Relative mRNA level was measured using qRT-PCR normalized to *GAPDH*, and the fold change (FC) between CCA and adjacent noncancerous tissues was obtained by using $2^{\Delta\Delta Ct}$ formula. (A) The percentage of frequency in differential gene expression was depicted in composite bar chart in which upregulation, unchanged, and downregulation were defined based on FC $\geqslant 2.0$, between 0.5 and 2.0, and $\leqslant 0.5$, respectively. (B) Box plot histograms depicted the median values of $2^{-dCt}$ relative to *GAPDH* mRNA expression in CCA (grey) and adjacent noncancerous tissues (white). Dots indicated outliers. Wilcoxon matched-pairs signed rank test was used to compare gene expression; $^{***}p < 0.0001$.

comprising seven upregulated (*RPSA*, *ITGAV*, *ITGA2*, *ITGA3*, *ITGA6*, *ITGB1*, and *ITGB4*) and one downregulated (*SDC2*) genes in CCA tissues were revealed. The expressions of eight genes were unaltered, including *SDC1*, *SDC4*, *ITGA1*, *ITGA4*, *ITGA7*, *ITGA9*, *ITGB2*, and *ITGB3*. However, of these 19 genes, *BCAM*, *DAG1*, and *SDC3* were exclusively upregulated in the TCGA-CCA cohort. This inconsistency might stem from the number of CCA and noncancerous paired-tissue samples obtained from the same donors. In the PCAWG project, 16 out of the 18 samples were collected from the same donors, while nine matched-samples were included in the TCGA-CCA data. Paired-sample RNA-seq data increase sensitivity for detecting the differential expression and the statistical power [26, 27]. Meta-analysis of multiple microarray datasets facilitates the examination of data from a number of independent studies, allowing the access of a huge number of samples to monitor global changes in gene expression, identify gene sets associated with interesting biological

characteristics, and classify new clinically and biologically important subclasses [28]. In this study, ten microarray datasets of CCA patients retrieved from the GEO database were used to investigate basal mRNA expression of laminins' cognate receptors. *ITGA1* and *ITGA9* were not consistently shared across microarray datasets and, therefore, were excluded from the analysis. Results from the analysis showed that five dysregulated genes (*SDC2*, *ITGAV*, *ITGA2*, *ITGB1*, and *ITGB4*) were shared with the two RNA-seq data; *SDC2* was downregulated, and the four latter genes were upregulated.

Integrin α2 (encoded by *ITGA2*) partners with only β1 subunit, while integrin αv (encoded by *ITGAV*) binds to diverse β subunits including β1 [12]. Integrin β1 (encoded by *ITGB1*) is a particularly promiscuous subunit that can partner with various α chains including α2 and αv, and seven out of nine laminin-interacting integrins contain β1 subunit – namely α1β1, α2β1, α3β1, α4β1, α6β1, α7β1, and α9β1 [12]. The elevated integrin β1, reported in numerous cancers: breast, ovarian, pancreatic, and skin; has impacts on cancer development [29, 30]. On the contrary, integrin β4, having a restricted ligand binding capacity, interacts only with integrin α6. Accumulating data have showed the elevated expression of α6β4 integrin and its roles in aggressive phenotypes of various carcinomas including CCA [10, 31]. Previously, we demonstrated the overexpression of integrin β4 in three (HuCCA-1, KKU-100, and KKU-213) of the four Thai patient-derived CCA cell lines compared with that of non-tumorigenic cholangiocyte MMNK-1, as well as the functions of integrin β4 as the main receptor in laminin-stimulated HuCCA-1 migration and adhesion [10]. Given the diversity in dimerization of the β1 and β4 subunits and their upregulation in several human malignancies, the overexpression of these two subunits from *in silico* studies was confirmed in the CCA tissues of Thai patients using qPCR analysis. The validated result demonstrated the prominent upregulation of *ITGB4* in 97% of the cases (29 out of 30), but the expression of *ITGB1* was not significantly altered. This finding was in line with our previous immunohistochemistry analysis that showed the absence of integrin β4 in all adjacent noncancerous tissues of Thai CCA patients. In addition, the elevation of this receptor was observed in 75% of the CCA cases (51 out of 68) that were associated with lymph node metastasis and short survival [10]. Taken together, the results suggested integrin β4 as a potential diagnostic biomarker to distinguish CCA patients from normal cases and a potential predictor of poor CCA prognosis.

In conclusion, the current study provides the baseline mRNA expression of laminins' cognate receptors in CCA tissues using bioinformatics analysis based on three transcriptomic datasets publicly available in the PCAWG, the TCGA, and the GEO databases. Moreover, the qPCR analysis validated the upregulated *ITGB4* in CCA tissues of Thai patients, suggesting the significance of understanding CCA cell-ECM interaction in the disease pathology and opening the door to use *ITGB4* as a diagnostic biomarker for CCA.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2022.049.

*Acknowledgements*: This work was supported by a grant from Mahidol University (Basic Research Funding: Fiscal year 2021). KI was supported by scholarship from Royal Golden Jubilee PhD Programme (RGJ) [scholarship no. PHD/0231/2553].

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**Appendix A. Supplementary data**

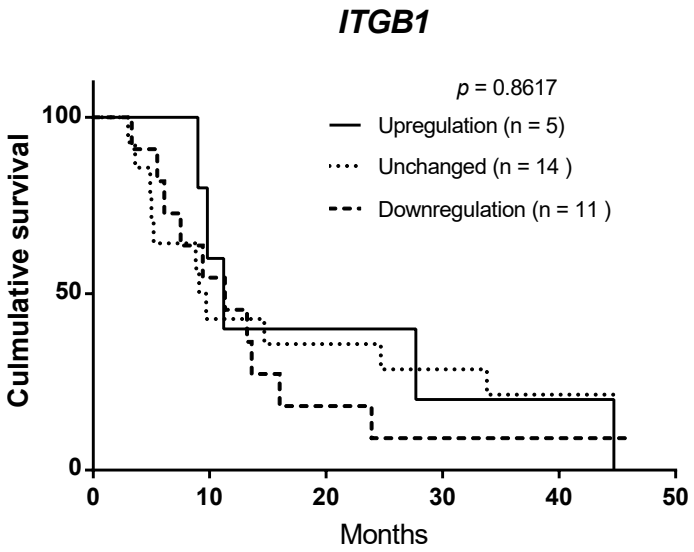


**Fig. S1** Kaplan-Meier survival plots of association of cholangiocarcinoma *ITGB1* expression levels and patients' survival post-liver resection.

**Table S1** Association of clinicopathological characteristics with *ITGB1* expression level in bile duct tissues of cholangiocarcinoma patients at the Srinagarind Hospital, Khon Kaen University, Khon Kaen Province, Thailand.

| Clinicopathological parameter | *n* | *ITGB1* | | | *p*-value |
|---|---|---|---|---|---|
| | | Upregulation 5 (16%) | Unchanged 14 (47%) | Downregulation 11 (37%) | |
| Age (years) | | | | | |
| $< 58$ | 13 | 3 | 6 | 4 | 0.790 |
| $\geqslant 58$ | 17 | 2 | 8 | 7 | |
| Gender | | | | | |
| Male | 19 | 3 | 9 | 7 | 1.000 |
| Female | 11 | 2 | 5 | 4 | |
| Histotype | | | | | |
| Non-invasive | 14 | 0 | 8 | 6 | 0.083 |
| Invasive | 16 | 5 | 6 | 5 | |
| Histotype ($n = 27$) | | | | | |
| Non-papilloma | 10 | 0 | 5 | 5 | 0.216 |
| Papilloma | 17 | 5 | 7 | 5 | |
| Histotype group ($n = 13$) | | | | | |
| Well-differentiation | 12 | 0 | 7 | 5 | 0.462 |
| Moderate-differentiation | 1 | 0 | 0 | 1 | |
| Metastasis | | | | | |
| Absent | 18 | 3 | 9 | 6 | 0.885 |
| Present | 12 | 2 | 5 | 5 | |
| Survival | | | | | |
| $< 1$ year | 17 | 3 | 8 | 6 | 1.000 |
| $\geqslant 1$ year (s) | 13 | 2 | 6 | 5 | |