Integrin β5 is an independent prognostic marker for intrahepatic cholangiocarcinoma in a Chinese population

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Abstract. Intrahepatic cholangiocarcinoma (ICC) is the second most common primary liver tumor and a major cause of cancer mortality worldwide. Integrin β5 (ITGB5) is considered to be involved in the intercellular signal transduction and regulation of tumorigenesis and development. The present study investigated the association between ITGB5 expression levels and the prognosis of ICC, as well as the effects of ITGB5 on the proliferation and invasion of ICC cells. RNA-sequencing transcriptomic profiling data of ICC samples were retrieved from The Cancer Genome Atlas (TCGA) and the Gene Expression Omnibus (GEO) databases. Tissue specimens from patients with ICC treated at Taizhou People's Hospital were collected and the ITGB5 expression levels were evaluated using immunohistochemical staining. The biological function of ITGB5 in ICC was investigated using Gene Ontology (GO), Gene Set Enrichment Analysis (GSEA) and *in vitro* experiments using HuCCT1 cells. After knocking down ITGB5 expression, cell proliferation was detected using Cell Counting Kit-8 assay, while cell invasion was assessed using Transwell assays. According to TCGA dataset, ITGB5 was highly expressed in ICC; however, there was no significant difference in prognosis between patients with high and low ITGB5 expression levels. High expression of ITGB5 was present in the tissues of patients with ICC from the GEO database, which was associated with poor prognosis. Survival analyses of the clinical data obtained in the present study revealed that high expression levels of ITGB5 in patients with ICC were associated with a reduced overall survival. GO and GSEA indicated that genes associated with ITGB5 were enriched in the extracellular matrix-receptor interaction and focal adhesion signaling pathways. Silencing ITGB5 inhibited the proliferation and invasion of ICC cells. In conclusion, ITGB5 may act as an essential regulator of ICC development and progression by influencing the proliferation and invasion of ICC cells. However, future studies with larger sample sizes are required to validate the role of ITGB5 in the prognosis of patients with ICC.

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Introduction

Intrahepatic cholangiocarcinoma (ICC) is a rare (0.79 cases per 100,000 individuals) but often fatal malignancy originating from the epithelium of the secondary bile duct and its branches (1,2). ICC is the second most common primary liver malignancy after hepatocellular carcinoma (HCC), and the incidence of ICC has increased globally over the last decades, whereas the incidence of perihilar cholangiocarcinoma and distal cholangiocarcinoma has decreased (3-8). Some of these changes are attributable to the alterations in disease classification, or to the more advanced diagnostic modalities that may identify early lesions and biliary malignancies that were previously undiagnosed (2). Furthermore, the increasing incidence of ICC may be associated with certain newly recognized strong risk factors, such as viral hepatitis, non-specific cirrhosis, nonviral chronic liver diseases and metabolic diseases (9). Due to the rarity, early metastasis and unclear symptoms of early ICC, only 10-15% of patients can undergo radical resection (10,11). The median overall survival (OS) of patients with ICC is 12-18 months, with the 5-year OS being rates <5% (12,13). Thus, it is necessary to elucidate the precise molecular mechanisms of ICC pathogenesis for predicting prognosis.

Integrins are a group of integral transmembrane heterodimers with numerous functions, including cell adhesion in the extracellular matrix (ECM) and acting as receptors for recognizing Arg-Gly-Asp (RGD) peptide motifs, laminin, snake venoms, viruses and other pathogens (14-17). Given the roles of integrins in multiple fundamental biological processes, the aberrant expression of integrin family members is linked to the prognosis of various types of cancer, including gastric cancer (18), breast cancer (19), pancreatic carcinoma (20) and colorectal cancer (21).

Integrin β5 (ITGB5), which associates with integrin αV (22), has been indicated to facilitate cancer cell migration, invasion and transforming growth factor β (TGF-β)-induced epithelial-mesenchymal transition (23). Lin *et al* (24) reported that ITGB5 promoted tumorigenesis in HCC by interacting with β-catenin. Furthermore, Wortzel *et al* (25) revealed that ITGB5 was enriched in liver metastatic pancreatic cancer exosomes. ITGB5 is a potential independent prognostic biomarker and therapeutic target for hepatitis B virus (HBV)-related HCC and may be useful for its diagnosis (26). These studies have indicated the potential role of ITGB5 in intercellular communication during tumor progression and metastasis. However, the role of ITGB5 in ICC remains largely unknown. The aim of the present study was to investigate the ITGB5 expression levels in ICC tissues and to examine whether the expression level of ITGB5 was associated with the prognosis of patients with ICC.

Materials and methods

Data processing of acquisition and identification of differentially expressed genes (DEGs). Microarray data for ICC were obtained from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). Two expression profiling datasets, GSE26566 (27) and GSE107943 (28), were obtained. The GSE26566 dataset contained 104 ICC samples and 6 normal samples. The GSE107943 dataset contained 30 ICC samples and 27 normal samples, as well as clinicopathological information regarding the tumor samples. All expression profiles were downloaded and processed using the R package of GEOquery (29). The transcriptome profiles of 32 ICC samples and 9 normal samples and clinical information of tumor samples were obtained from The Cancer Genome Atlas (TCGA) database (https://cancergenome.nih.gov/) and analyzed using the R package of TCGAbiolinks (30). The GSE26566 and TCGA datasets were analyzed separately as volcano maps using GraphPad Prism 9 (GraphPad Software; Dotmatics). Log_2 fold-change (FC)>1 and P<0.05 were defined as the screening thresholds. Common DEGs of the GSE26566 and TCGA datasets were obtained through the TBtools (31). For the definition of high or low expression levels of ITGB5 in the GSE107943 and TCGA datasets, the expression of ITGB5 was divided into two groups according to the survival status of patients with ICC, and separate receiver operating characteristic curves were obtained to determine the ITGB5 cut-off value with area under the curve >0.8 and P<0.05.

Patient tissues. The present retrospective study on patient tissues and data was approved by the Medical Ethics Committee of Taizhou People's Hospital (approval no. KY 2020-091-01), and was conducted according to the Declaration of Helsinki. Written informed consent was received from each patient at the time of surgery. Surgical resection for ICC was used to treat 34 patients in Taizhou People's Hospital (Taizhou, China) between January 2014 and December 2020 (Table I). All specimens were obtained from the Department of Pathology of Taizhou People's Hospital, and were histologically diagnosed in accordance with the World Health Organization criteria (32). Clinical features were extracted from patient medical records. Tumor stages were classified according to the 8th American Joint Cancer Committee tumor-node-metastasis (TNM) classification (stages I-IV) (33).

Gene function enrichment analysis. Gene Ontology (GO) is widely used in bioinformatics, and covers three aspects of biology, namely biological processes, molecular functions and cellular components (34). Kyoto Encyclopedia of Genes and Genomes (KEGG) is a set of high-throughput genes and protein pathways (35). Metascape is an online analysis tool suite with the function of annotations and analyses (36). GO and KEGG pathway enrichment analyses were performed for the upregulated DEGs using Metascape analysis. All significant GO and KEGG enrichment results were visualized with the bubble chart of GraphPad Prism 9 (GraphPad Software; Dotmatics).

Protein-protein interaction (PPI) network and functional annotations of ITGB5. The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) online database (http://string-db.org) could predict and trace out the PPI network. The top 10 interacting genes associated with ITGB5 were obtained using STRING. Cytoscape version 3.9.0, a free visualization software, was used to visualize the PPI network (37). The 210 genes interacting with ITGB5, as determined using the STRING online database (10 genes) and Gene Expression Profiling Interactive Analysis of GEPIA (38) (200 genes), were all inputted into the Metascape (36) for further functional annotations and analyses. Gene Set Enrichment Analysis (GSEA) version 4.2.3 (39), a free gene analysis software, was used to reveal the functional pathways of ITGB5 in ICC, using transcriptional data from the GSE26566 dataset. A 1,000 permutation test, nominal (NOM) P<0.05 and false discovery rate (FDR) q<0.25 were used as the screening criteria to identify the most significantly involved pathways. Patients were divided into two groups according to the median ITGB5 mRNA expression level. GSEA was performed to determine whether the identified sets of genes exhibited significant differences between the two groups based on the normalized enrichment score (NES) and FDR. Based on correlation coefficients, pathway analysis associated with ITGB5 using GSEA was implemented. The aforementioned three methods were validated against each other to determine the most relevant pathway.

Cell lines and culture. Human cholangiocarcinoma HuCCT1 cells were purchased from the Shanghai Cell Bank of The Chinese Academy of Science. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS) (Invitrogen; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37˚C in a humidified environment containing 5% CO_2.

Immunohistochemical (IHC) staining. Paraffin-embedded sections of tissues were deparaffinized, hydrated and incubated with 0.3% hydrogen peroxide to block endogenous peroxidase. Microwave heating (microwave oven for 30 min at 250 W) was used for antigen retrieval. The sections were first incubated in a 2% bovine serum albumin buffer (Sigma-Aldrich; Merck KGaA) at 37˚C for 30 min and then at 4˚C overnight with anti-ITGB5 rabbit polyclonal antibody (1:100; cat. no. ab15459; Abcam). For the antibody binding, The sections were then washed three

Table I. Clinical characteristics of patients with intrahepatic cholangiocarcinoma.

Clinicopathological characteristic	N (%)
Sex	
Male	21 (62)
Female	13 (38)
Age, years	
≤65	20 (59)
>65	14 (41)
Histological grade	
High	5 (15)
Medium	15 (44)
Low	14 (41)
TNM stage	
I	7 (21)
II	10 (29)
III	7 (21)
IV	10 (29)
Serum CA19-9 levels, U/ml	
≤37	4 (12)
>37	30 (88)
ITGB5 expression	
High	22 (65)
Low	12 (35)

TNM, tumor-node-metastasis; CA19-9, carbohydrate antigen 19-9; ITGB5, integrin β5.

times with 0.5% Tween, 0.1 M Tris-base, 0.9% NaCl, (TBS-T; pH 7.6) for 5 min each wash and incubated in biotinylated goat anti-rabbit IgG (1:100; cat. no. ab172730; Abcam) at 37˚C for 30 min. Positive reactions were visualized using diaminobenzidine solution followed by counterstaining with hematoxylin at room temperature for 8 min. Tissue sections were observed using an AX10-Imager A1 light microscope (Zeiss GmbH), and all images were captured using AxioVision microscopy software (version 4.7; Zeiss GmbH). All IHC staining was independently evaluated by two experienced gastrointestinal pathologists. IHC scores were calculated by multiplying the intensity of staining (0: Negative; 1: Light yellow; 2: Yellowish brown; and 3: Brown) by the percentage of positive cells (1: <5%; 2: 6-25%; 3: 26-70%; and 4: >70%), and finally interpreted as high or low expression levels. If the final score was ≥4, the ITGB5 expression level was considered high; otherwise, it was considered low.

Western blot analysis. Whole-cell lysates were prepared by lysing HuCCT1 ($2x10^6$ cells/ml) pellets in RIPA lysis buffer. Following centrifugation at 1,000 x g for 30 min at 4˚C to remove all debris, and the protein levels were estimated using a Super-Bradford Protein Assay kit (CoWin Biosciences Co., Ltd.), according to the manufacturer's protocol. Each 40-μg aliquot of total protein was loaded on a 10% SDS-PAGE gel (25 μg) and separated at 100 V for 1.5 h. After electrophoresis, the proteins were transferred onto PVDF membranes (EMD Millipore) and then blocked with 5% skimmed milk for 60 min at room temperature. After washing with TBST three times, membranes were co-incubated with the primary antibodies against ITGB5 (1:500 dilution; cat. no. ab184312) and β-actin (1:1,000 dilution; cat. no. ab115777; both from Abcam) overnight at 4˚C in TBST. After incubation with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:5,000 dilution; cat. no. BA1055; Beyotime Institute of Biotechnology) in TBST at room temperature for 60 min, bands were detected using BeyoECL Plus (Beyotime Institute of Biotechnology) and captured using an Image Quant LAS-4000 (FUJIFILM Wako Pure Chemical Corporation). The expression of ITGB5 protein was normalized to β-actin expression.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA from harvested cells was extracted using an RNA isolation kit with genomic DNA filter columns (BioTeke Corporation) according to the manufacturer's protocol. After RNA quantification and quality control using spectrophotometry with the optical density (OD) OD260/OD280 ratio controlled at 1.8-2.0, RNA samples were reverse transcribed into cDNA using a reverse transcriptase kit (Takara Biotechnology Co., Ltd.), followed by PCR with SYBR® Green RT-PCR Master mix (Takara Bio, Inc.) according to the manufacturer's protocols. The relative levels of target gene mRNA transcripts to the control β-actin in individual samples were determined in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.) under the following thermocycling conditions: 50˚C for 2 min, 9˚C for 10 min, 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. Data were normalized to the control β-actin and analyzed using the $2^{-\Delta\Delta Cq}$ method (40). Samples were assayed in triplicate in three independent experiments. The primers for the amplification of the indicated genes were as follows: ITGB5, forward 5'-ACCT GGAACAACGGTGGAGA-3' and reverse 5'-AAAAGATGC CGTGTCCCCAA-3'; and β-actin, forward 5'-CAAGAGATG GCCACGGCTGCT-3' and reverse 5'-TCCTTCTGCATCCTG TCGGCA-3'.

Small interfering RNA (siRNA) transfection. siRNA against ITGB5 and a negative control siRNA were designed and synthesized by Shanghai GenePharma Co., Ltd. HuCCT1 cells were transfected with siRNA (800 μg/ml) using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were plated in six-well plates at a density of $2x10^6$ cells/well. Lipofectamine® 3000 and siRNA were mixed together in Opti-MEM (Invitrogen; Thermo Fisher Scientific, Inc.). The cell culture medium was replaced with Opti-MEM at 37˚C for 6 h. The cells were cultured in normal medium for 48 h before the subsequent experiments. The ITGB5 siRNA sequences were: Sense 5'-GGAGGUUACUGAAUGACAAAC-3' and antisense 5'-UUGUCAUUCAGUAACCUCCUA-3'. The sequences of the control non-targeting siRNA were: Sense 5'-UUCUCCGAA CGUGUCACGUTT-3' and antisense 5'-ACGUGACACGUU CGGAGAATT-3'. Knocked down expression was confirmed using RT-qPCR or western blotting.

Cell Counting Kit-8 (CCK-8) and Transwell assays. CCK-8 assay (Nanjing KeyGen Biotech Co., Ltd.) was used to evaluate cell proliferation and viability. Cells (~$1x10^5$) were seeded in

100 μl DMEM per well in a 96-well plate. Subsequently, 100 μl CCK-8 solution was added to each well and incubated at 37˚C for additional 2 h. The absorbance at 450 nm was measured on a spectrophotometric plate reader. Each group was repeated in three different wells.

The invasiveness of cholangiocarcinoma cells was detected using 24-well Transwell plates (8-μm pore size; Corning, Inc.). The bottom of each well insert was precoated with 50 μg Matrigel (BD Biosciences) to simulate matrix barriers at 37˚C for 4 h. Cells ($1x10^4$) in 200 μl serum-free medium were added to each upper chamber, and the lower compartments were filled with 600 μl medium containing 10% FBS. Following incubation for 24 h at 37˚C, the invasive cells in the lower chamber were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet at room temperature for 15 min. The stained cells were counted under a light microscope (Olympus Corporation) in five random fields.

Statistical analysis. Statistical analyses were performed using SPSS version 26.0 (IBM Corp.) and GraphPad Prism version 9 (GraphPad Software; Dotmatics). For the tumor tissue and the normal tissue adjacent to the tumor samples in the GSE107943 dataset, the ITGB5 mRNA expression levels were analyzed using a paired Student's t-test. Two-sided Fisher's exact test was used to reveal the association between the expression levels of ITGB5 and clinicopathological features. Clinicopathological variables with P<0.05 in univariate Cox regression analysis were further analyzed using multivariate Cox regression. Survival data were analyzed using Kaplan-Meier survival curves with a log-rank test. Experimental data (≥3 independent replicates) are expressed as the mean ± standard deviation. One-way ANOVA followed by Tukey's post-hoc test was used to reveal the invasiveness of tumor cells, while two-way ANOVA followed by Tukey's post-hoc test was used to evaluate the proliferation and viability of tumor cells. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification and analysis of DEGs, and upregulation of ITGB5 in ICC tissues. The GSE26566 and TCGA datasets were analyzed separately as volcano maps using GraphPad Prism 9 (GraphPad Software; Dotmatics). A total of 4,695 DEGs (log_2 FC>1, corrected P<0.05) were obtained, including 2,662 significantly upregulated and 2,033 significantly downregulated DEGs. After standardization of the microarray results, 2,606 DEGs from the GSE26566 dataset were obtained, including 1,515 upregulated genes and 1,091 downregulated genes. A total of 1,370 common DEGs (777 upregulated and 593 downregulated) in the two datasets were obtained through the TBtools (31) (Fig. 1A and B). In addition to those published genes of minichromosome maintenance complex component 6 (MCM6) (41) and tripartite motif containing 59 (TRIM59) (42), a small number of significant DEGs were highlighted, including ITGB5. In TCGA and GSE26566 datasets, the volcano map indicated that ITGB5 was significantly overexpressed in ICC (Fig. 1C and D), which was consistent with the ITGB5 mRNA expression levels in ICC in the GSE107943 dataset (Fig. 1E). These results indicated that ITGB5 was significantly overexpressed in ICC tumor tissue compared with adjacent normal tissue. IHC analysis of tumor tissue samples of 34 patients with ICC revealed that the ITGB5 expression levels were significantly increased in 12 patients (Fig. 2).

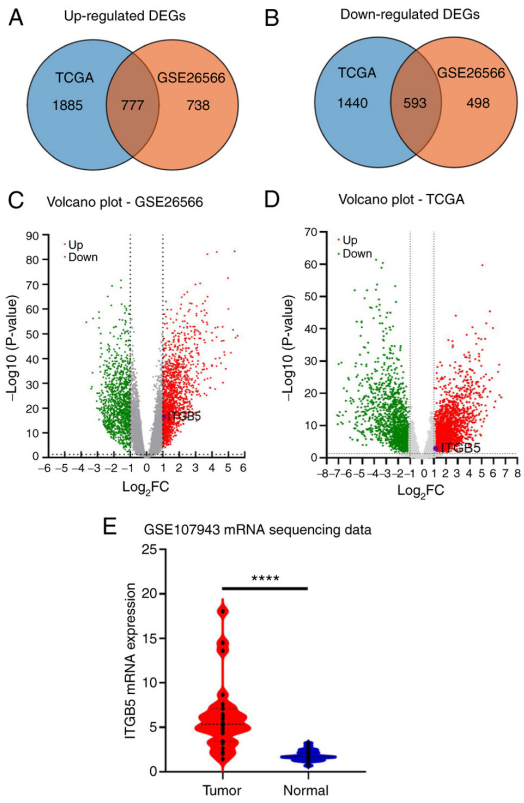

Figure 1. Identification and analysis of DEGs in ICC. (A) Overlap of 777 common upregulated DEGs among the GSE26566 and TCGA datasets. (B) Overlap of 593 common downregulated DEGs among the GSE26566 and TCGA datasets. Differential expression of genes in tumor and normal samples in the (C) GSE26566 and (D) TCGA datasets. Upregulated genes with adjusted P<0.05 and log2 FC>1 are marked in red. Downregulated genes with adjusted P<0.05 and log_2 FC≤1 are marked in green. The gray points represent genes with no significant difference. ITGB5 is marked in violet and was upregulated in ICC. (E) ITGB5 mRNA expression levels in ICC and adjacent normal tissues in the GSE107943 dataset. ****P<0.001. DEGs, differentially expressed genes; ICC, intrahepatic cholangiocarcinoma; TCGA, The Cancer Genome Atlas; FC, fold-change; ITGB5, integrin β5.

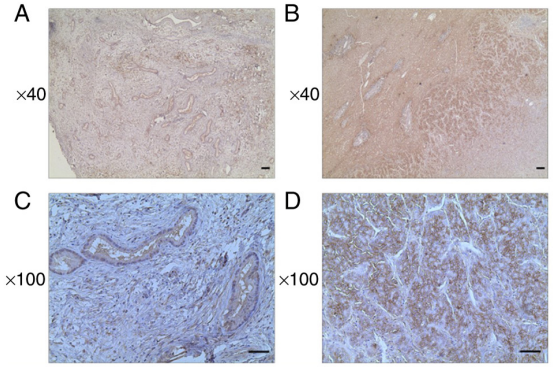

Figure 2. Expression levels of ITGB5 in ICC. (A) Low expression levels of ITGB5 in ICC tissues and (B) strong staining of ITGB5 in ICC tissues at x40 magnification. (C) Low expression levels of ITGB5 in ICC tissues and (D) strong staining of ITGB5 in ICC tissues at x100 magnification. Scale bar, 50 μm. ITGB5, integrin β5; ICC, intrahepatic cholangiocarcinoma.

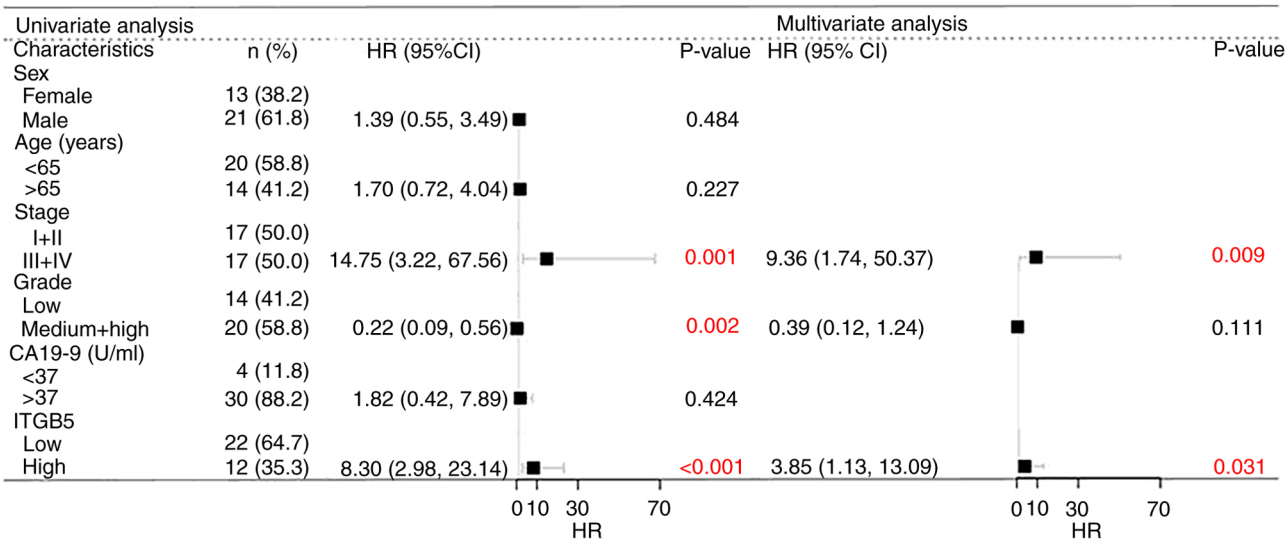

Figure 3. Univariate and multivariate Cox regression analyses of patients with intrahepatic cholangiocarcinoma. The hazard ratio of univariate and multivariate Cox regression analysis was visualized using a forest plot. HR, hazard ratio; CI, confidence interval; CA19-9, carbohydrate antigen 19-9; ITGB5, integrin β5.

Association of ITGB5 with clinicopathological characteristics and OS in patients with ICC. Two-sided Fisher's exact test of clinicopathological characteristics analysis indicated that the expression of ITGB5 was significantly associated with histological grade and TNM stage, but not with clinicopathological indicators such as sex, age or serum carbohydrate antigen (CA)19-9 level (Table II). In addition, to the best of our knowledge, jaundice had no effect on ITGB5 expression levels. Therefore, there was no further discussion regarding the association between ITGB5 and jaundice. The present study followed up 34 patients with ICC, and the difference in survival between the high and low ITGB5 expression groups was investigated using the Kaplan-Meier method. Multiple factors affecting survival in patients with ICC were analyzed using the Cox model. Univariate analysis indicated that sex, age and serum CA19-9 level did not significantly affect the survival of patients with ICC, while a low histological grade, late TNM stage and high expression levels of ITGB5 were risk factors for a reduced survival rate. Multivariate analysis suggested that a low histological grade was not an independent risk factor affecting the survival of patients with ICC, while high expression levels of ITGB5 and a late TNM stage were independent risk factors for a reduced survival rate (Fig. 3).

The patients in the high ITGB5 expression level group had a mean survival of 4.31±1.17 months, which was significantly reduced compared with that of patients in the low ITGB5 expression level group (44.23±9.39 months; P<0.001; Fig. 4A). Survival curves were produced using the ICC clinical data for the GSE107943 (Fig. 4B) and TCGA (Fig. 4C) datasets. The GSE107943 dataset indicated that patients in the high ITGB5 expression level group had a mean OS of 78.89±10.21 months, which was significantly increased compared with that of patients in the low ITGB5 expression level group (25.90±3.93 months; P=0.005). However, the results of TCGA showed no significant difference in the mean OS time between the high and low ITGB5 expression level groups.

Gene enrichment analysis. To elucidate the effect of the screened differential genes on ICC, gene enrichment analysis was performed using Metascape, which included GO and KEGG pathway enrichment analyses. Since the ITGB5 expression level was high, enrichment analysis was performed on upregulated genes. Through GO enrichment analysis of the upregulated genes, numerous enriched gene sets were revealed. In terms of the biological processes, they were enriched in 'regulation of cell adhesion', 'extracellular matrix organization', 'extracellular structure organization', 'external encapsulating structure organization' and 'mitotic cell cycle process' (Fig. 5A). In terms of the cellular components, they were significantly enriched in 'extracellular matrix', 'external encapsulating structure', 'collagen-containing extracellular matrix', 'basement membrane' and 'focal adhesion' (Fig. 5B). In terms of the molecular function, they were mainly enriched in 'extracellular matrix structural constituent', 'cell adhesion molecule binding', 'structural molecule activity', 'kinase binding' and 'extracellular matrix structural constituent conferring tensile strength' (Fig. 5C). The functional significance of differential mRNAs in the development of ICC was analyzed through KEGG pathway analysis. The results of KEGG analysis revealed that upregulated genes were significantly enriched in 'ECM-receptor interaction', 'focal adhesion', 'human papillomavirus infection', 'pathways in cancer' and 'protein digestion and absorption' (Fig. 6).

Prediction of interaction networks of ITGB5 and enrichment analysis of genes co-expressed with ITGB5. A PPI network for ITGB5 was constructed using the STRING online database. The node representing ITGB5 was connected to the nodes of other genes in terms of co-expression and physical interactions. The PPI network of the top 10 genes was visualized using Cytoscape (Fig. 7A). For biological pathway analysis of genes co-expressed with ITGB5, the top 200 genes strongly correlated with ITGB5 from GEPIA and 10 genes of PPI networks of ITGB5 were all inputted into Metascape for functional annotations and analyses.

Table II. Association between ITGB5 expression levels and intrahepatic cholangiocarcinoma characteristics.

Characteristic	ITGB5		P-value
	Low	High	
Sex			0.727
Male	13	8	
Female	9	4	
Age, years			0.163
≤65	15	5	
>65	7	7	
Histologic grade			0.014[a]
High	4	1	
Medium	13	2	
Low	5	9	
TNM stage			0.015[a]
I	7	0	
II	8	2	
III	4	3	
IV	3	7	
Serum CA19-9, U/ml			0.556
≤37	3	1	
>37	19	11	

[a]P<0.05. CA19-9, carbohydrate antigen 19-9; ITGB5, integrin β5; TNM, tumor-node-metastasis.

The genes associated with biological pathways were mainly enriched in 'focal adhesion', 'human papillomavirus infection' and 'ECM-receptor interaction' (Fig. 7B). Based on the GSE26566 dataset, pathway analysis of genes positively associated to ITGB5 was also performed in GSEA. The genes associated with biological pathways were mainly enriched in 'focal adhesion' (NES=1.96, NOM P=0.005 and FDR q<0.127), 'adherens junction' (NES=1.95, NOM P<0.0001 and FDR q=0.070) and 'ECM-receptor interaction' (NES=1.85, NOM P=0.011 and FDR q=0.160) (Fig. 8A-C). To identify the signaling pathways activated by the differential upregulation of ITGB5 expression in ICC, GSEA of samples with low and high ITGB5 expression level based on the GSE26566 dataset was performed. 'Focal adhesion' (NES=2.23, NOM P<0.0001 and FDR q=0.000683), 'ECM-receptor interaction' (NES=2.13, NOM P<0.0001 and FDR q=0.0048), and 'lysosome' (NES=1.96, NOM P=0.0019 and FDR q=0.0360) were significantly enriched in the high ITGB5 expression level sample (Fig. 8D-F). The aforementioned three methods were validated against each other to determine the most relevant pathway. The results revealed that ITGB5 may be involved in the progression of ICC by regulating ECM-receptor interaction and focal adhesion pathways. ECM-receptor interaction and focal adhesion signaling pathways were also the most significantly enriched for upregulated DEGs using Metascape analysis, which is consistent with the aforementioned results.

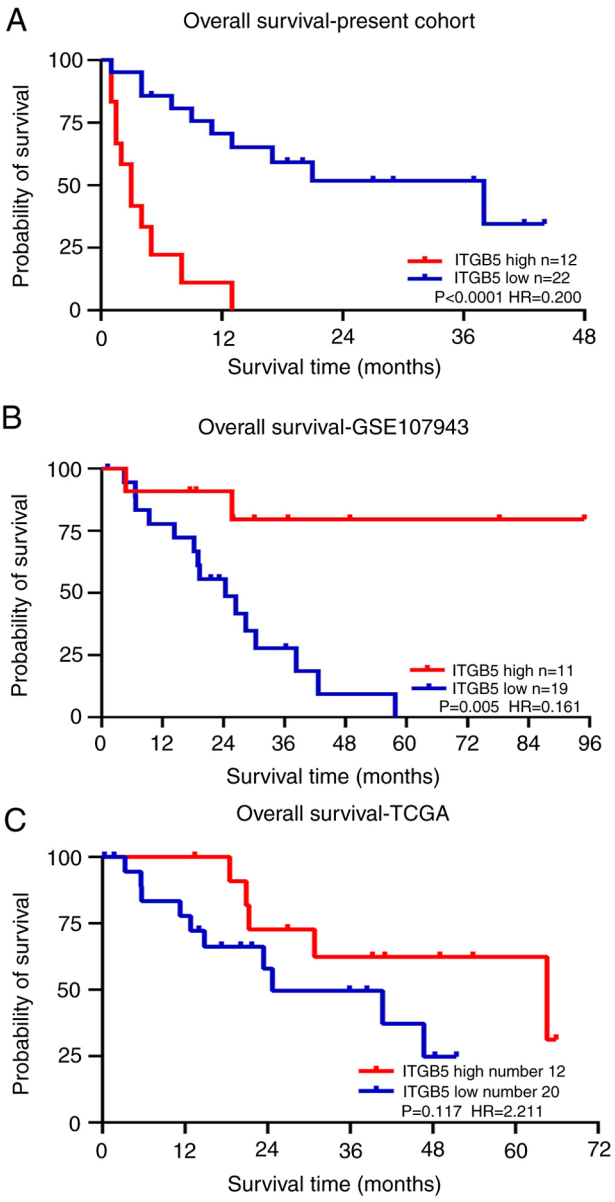

Figure 4. Evaluation of the prognostic value of ITGB5 in patients with intrahepatic cholangiocarcinoma from the present cohort, and from the GSE107943 and TCGA datasets. (A) According to the present cohort, high ITGB5 expression levels were associated with a reduced OS compared with that of low ITGB5 expression levels. (B) The results from the GSE107943 dataset indicated that patients with high ITGB5 expression levels had an increased OS compared with that of patients with low ITGB5 expression levels. (C) The results from TCGA dataset indicated no statistical difference in the mean survival time between the ITGB5 high- and low-expression groups. P<0.05 was considered to indicate a statistically significant difference. ITGB5, integrin β5; TCGA, The Cancer Genome Atlas; HR, hazard ratio; OS, overall survival.

Knockdown of ITGB5 inhibits the proliferation and invasion of ICC cells. As ITGB5 was highly expressed in certain patients with ICC and was significantly correlated with prognosis, the functional roles of ITGB5 in human ICC cells were investigated. Western blotting and RT-qPCR were used to test the efficiency of ITGB5 silencing in HuCCT1 cells. The ITGB5 mRNA and protein expression levels were reduced after transfection with ITGB5-specific siRNA (Fig. 9A and B). Transwell invasion assays were performed in HuCCT1 cells after downregulation of ITGB5. The invasiveness of ICC

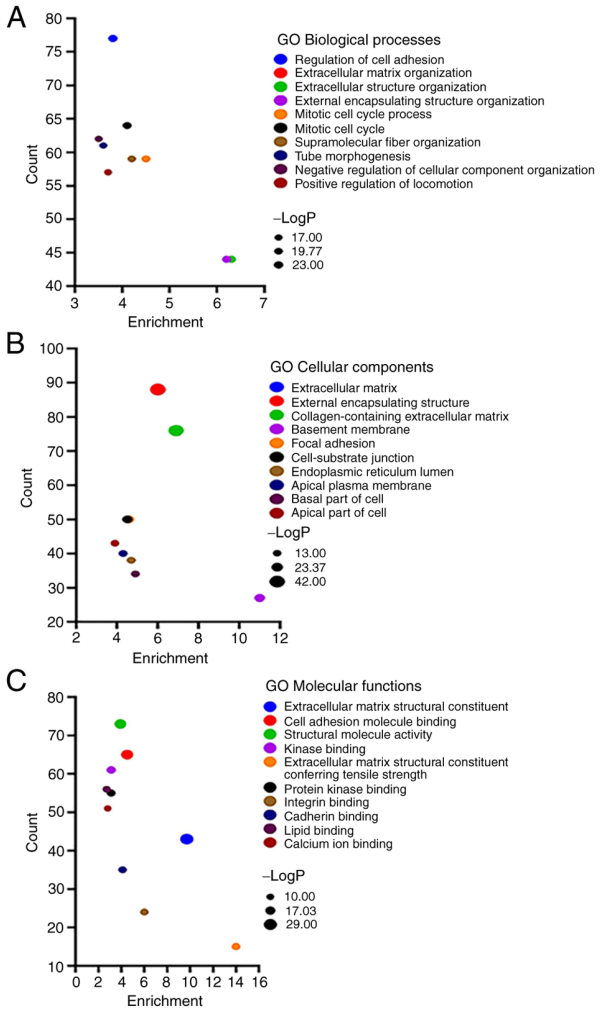

Figure 5. Significant GO enrichment pathways of differentially expressed genes in intrahepatic cholangiocarcinoma. (A) Biological processes terms of GO. (B) Cellular components terms of GO. (C) Molecular functions terms of GO. GO, Gene Ontology.

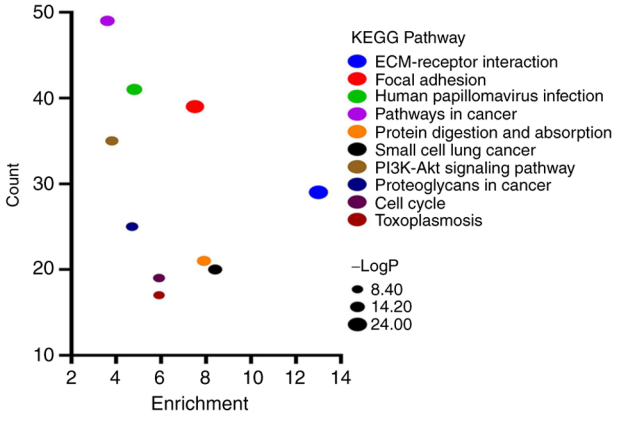

Figure 6. Significant Kyoto Encyclopedia of Genes and Genomes enrichment pathways of differentially expressed genes in intrahepatic cholangiocarcinoma. ECM, extracellular matrix.

cells was significantly reduced by ITGB5 silencing (Fig. 9C). CCK-8 assays were used to determine the effects of ITGB5 on the viability of ICC cells. The results demonstrated that

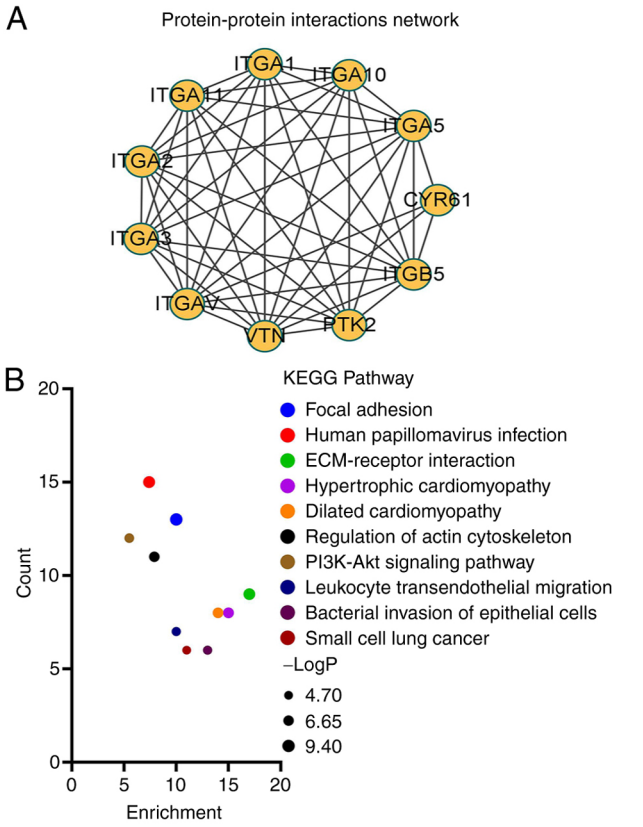

Figure 7. Protein-protein interaction network and KEGG pathways analysis of ITGB5 and associated genes. (A) The interaction network of ITGB5 and its associated genes was visualized by Cytoscape. (B) Top 10 significant KEGG pathways of ITGB5 and its associated genes were visualized by Metascape. ECM, extracellular matrix; KEGG, Kyoto Encyclopedia of Genes and Genomes; ITGB5, integrin β5; ITGA1, integrin α1; VTN, vitronectin; PTK2, protein tyrosine kinase 2; CYR61, cysteine-rich angiogenic inducer 61.

ITGB5 silencing in HuCCT1 cells significantly reduced cell viability 72 h after transfection (Fig. 9D).

Discussion

Given the incidence of ICC increasing from 0.44 to 1.18 cases per 100,000 (2) and its high morbidity, early prediction of prognosis is an arduous and urgent task. Identifying ICC-specific diagnostic biomarkers has been a focus among numerous studies, which is associated with advances in omics technologies. In the past decade, efforts have been conducted to elucidate the molecular pathogenesis of cholangiocarcinoma, particularly ICC, through the application of multi-omics approaches, including genomic, epigenomic, transcriptomic and metabolomic analyses (43,44). It has been reported that SMAD4 expression levels are associated with the prognosis of patients with ICC (45). It has been reported that ITGA6 is highly expressed in ICC and promotes the proliferation and invasion of ICC cells (46). ITGB5 is a potential independent prognostic biomarker and therapeutic target for patients with HBV-related HCC. Previous studies have demonstrated that ITGB5 is a prognostic biomarker and new therapeutic target for human pancreatic (47), breast (48), gastric (49) and ovarian (50) cancer, as well as glioblastoma (51). The present study revealed increased ITGB5 mRNA and protein expression levels in

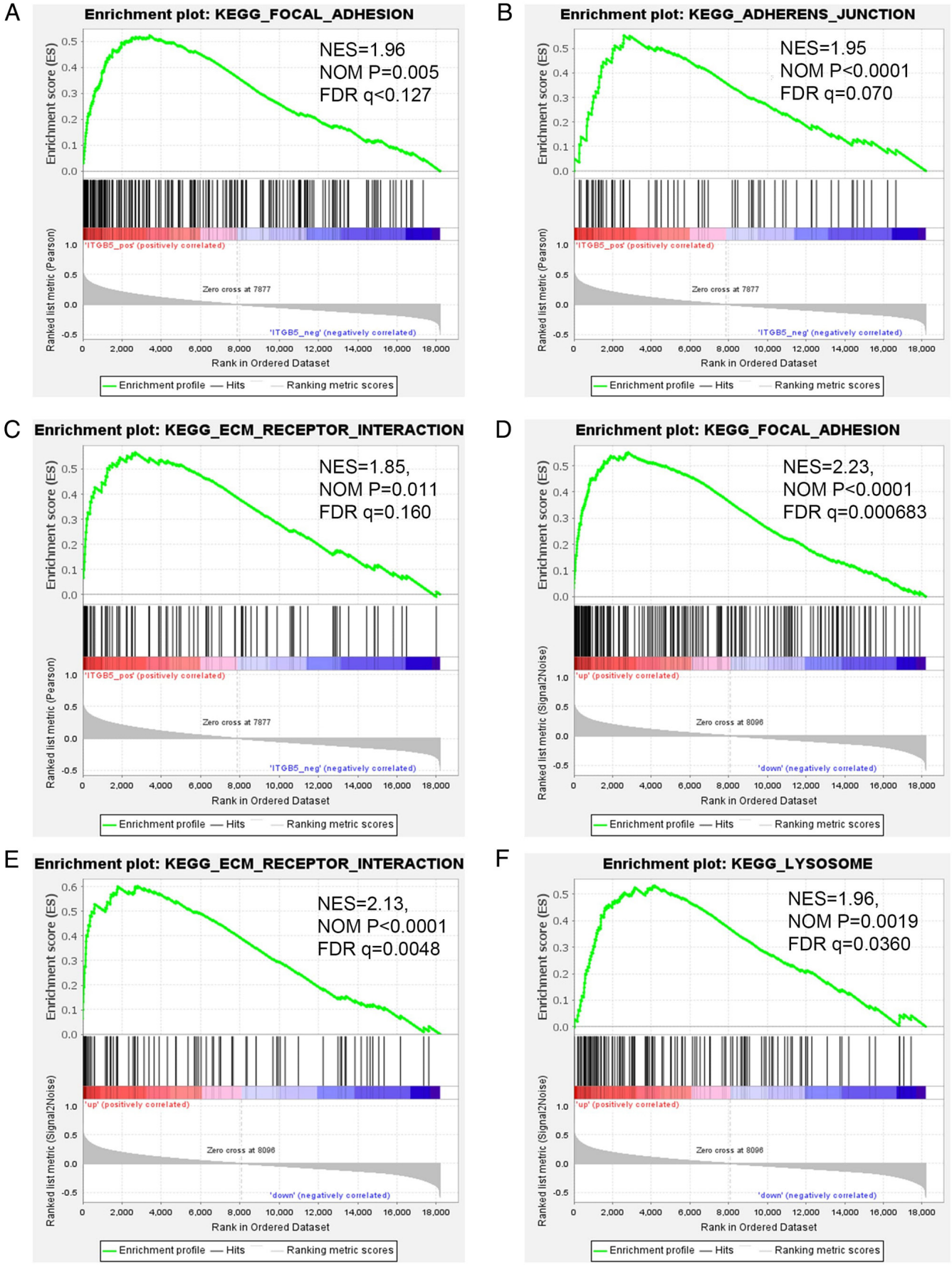

Figure 8. GSEA-KEGG pathway analysis of ITGB5-associated genes in the GSE26566 dataset. Top three significant GSEA-KEGG pathways of the ITGB5-associated genes in the GSE26566 dataset, namely (A) focal adhesion, (B) adherens junction and (C) ECM receptor interaction. Top three significant GSEA-KEGG pathways of high ITGB5 expression level samples in the GSE26566 dataset, namely (D) focal adhesion, (E) ECM receptor interaction and (F) lysosome. ECM, extracellular matrix; GSEA, gene set enrichment analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; ITGB5, integrin β5.

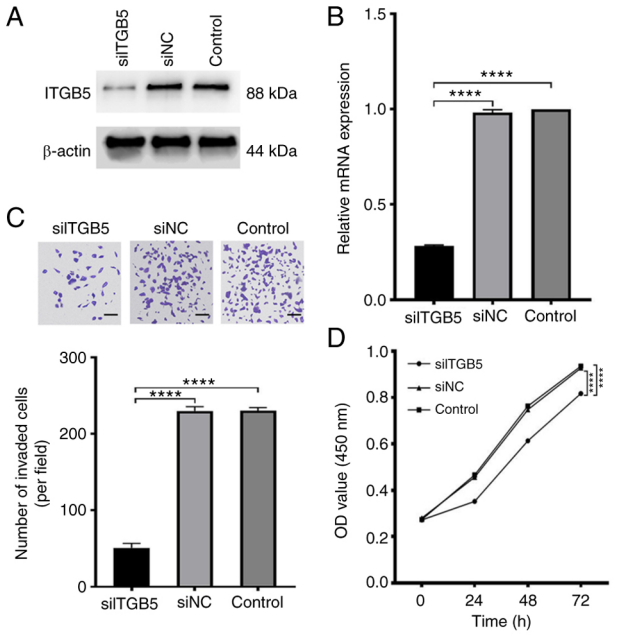

Figure 9. Knocked down ITGB5 inhibits the proliferation and invasion of HuCCT1 cells. The (A) protein and (B) mRNA expression levels of ITGB5 were detected using western blotting and reverse transcription-quantitative polymerase chain reaction, respectively, after a 48-h incubation of the siRNA-lipid complex. β-actin was used as an internal control for normalization. (C) The invasion ability of HuCCT1 cells transfected with ITGB5 siRNA was measured by Transwell assays. Scale bar, 20 μm. (D) The viability of HuCCT1 cells transfected with ITGB5 siRNA was detected by Cell Counting Kit-8 assay. ****P<0.001. ITGB5, integrin β5; siRNA, small interfering RNA; NC, negative control; OD, optical density.

ICC tissues, and the upregulation of ITGB5 was associated with the late TNM stage and low histological grade. Another novel finding of the present study was that high ITGB5 levels were independently correlated with a reduced survival rate in patients with ICC. Therefore, whether ITGB5 can predict the prognosis of patients with ICC requires further investigation.

Deregulation of integrin signaling is associated with carcinogenic effects in a number of malignancies. For example, in pancreatic cancer, ITGB4 is associated with epithelial-mesenchymal transition. Overexpression of ITGB4 promotes pancreatic carcinogenesis and regulates the MEK1-ERK1/2 signaling pathway (52). ITGB6 promotes the invasion of various cancer cells, including colorectal and pancreatic cancer, through the ERK and TGF signaling pathways, which promote matrix metalloproteinase activation (53,54). As for ITGB5, Lin *et al* reported that it was highly expressed in HCC, and microRNA-185 regulated the expression of β-catenin in an ITGB5-dependent manner, and affected the proliferation and migration of HCC cells (24). Tumor cells with knocked out ITGB5 led to a reduced disease burden and a prolonged survival in mice, demonstrating the contribution of ITGB5 to pancreatic ductal adenocarcinoma progression (55). A previous study demonstrated that exosomal ITGB5 regulated liver tropism, which was associated with liver metastasis in a number of malignancies, including colorectal, pancreatic and gastric cancer (56). In the present study, to investigate the function of ITGB5 in ICC, knockdown experiments using siRNAs were performed, which demonstrated that HuCCT1 cell proliferation and invasion were reduced by ITGB5 depletion. For patients with ICC, TCGA dataset indicated no significant difference in the prognosis of patients with high or low ITGB5 expression levels. However, high ITGB5 expression levels reflected an increased OS rate in the GSE107943 dataset, while high ITGB5 expression levels reflected a reduced OS rate in the data of the present study. Due to the rarity of ICC, studies on ICC often have small cohort sizes, which may contribute to the aforementioned observed difference in OS. Ethnic heterogeneity and differences in TNM stages might be other factors explaining the differences in prognosis. The patients with ICC in the cohort of the present study were of Chinese ethnicity, and the majority exhibited TNM stages III and IV, while the patients with ICC in the GES107943 dataset were South Koreans in ethnicity and mainly exhibited TNM stages I and II.

To investigate the signaling pathways contributing to ICC progression, the current data were processed through bioinformatics methods to obtain additional information regarding ITGB5 and its co-expressed genes. The aforementioned methods were validated against each other to determine the most relevant pathway. The results revealed that ITGB5 might be involved in the progression of ICC by regulating the ECM-receptor interaction and focal adhesion pathways. The two aforementioned pathways were the most significantly enriched for upregulated DEGs of ICC using Metascape analysis, which is consistent with a previous study (57). These results suggest that ITGB5 promotes tumor cell proliferation and migration through ECM-receptor interaction and focal adhesion signaling pathways, which may lead to the poor survival of patients with ICC. A number of studies have demonstrated the involvement of ECM-receptor interaction in the development and formation of metastases in various tumors, including breast and lung cancer, as well as glioma, through its regulation of integrin expression levels (58-61). The focal adhesion signaling pathway via integrin has an effect on the regulation of the ECM, cell migration and tumor microenvironment (62). The focal adhesion pathway facilitates the interplay between tumors and the ECM, serving as a crucial link connecting them (63). However, to the best of our knowledge, although a number of studies have explored the association between ITGB5 and the ECM-receptor interaction and focal adhesion signaling pathway in gastric cancer (64,65), no comprehensive prognostic analysis of ECM-receptor interaction and focal adhesion-associated genes in ICC has been conducted to date. Signaling pathways associated to ITGB5 that affect ICC survival will be the next aim of our future research.

There were a number of limitations in the present study that should be addressed. Firstly, using the normal bile duct tissue located next to the ICC tumor as the negative control to compare the changes in ITGB5 expression levels using IHC would have improved the present study. However, the present results only revealed high and low ITGB5 expression levels, which means that the comparison of ITGB5 in ICC tumor tissue and adjacent normal tissue is inadequate at the protein level. Secondly, the sample size was insufficient for the tumor size and macroscopic analysis of clinical features. Although T stage encompasses more comprehensive information than tumor size, potential bias may arise in the results of

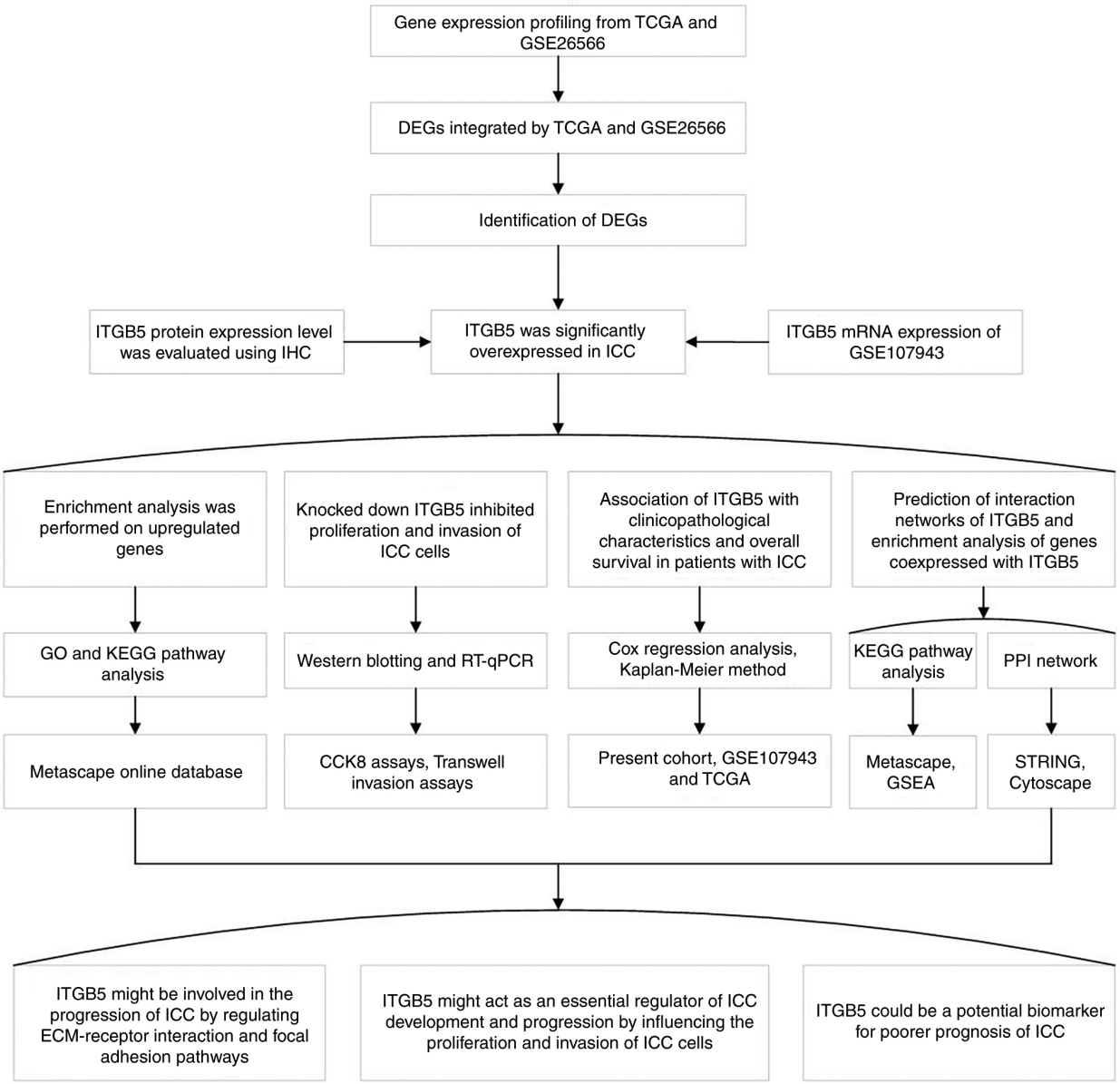

Figure 10. Flowchart of the present study describing the main methods used and the results obtained. TCGA, The Cancer Genome Atlas; DEG, differentially expressed gene; ITGB5, integrin β5; IHC, immunohistochemistry; ICC, intrahepatic cholangiocarcinoma; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; PPI, protein-protein interaction; CCK-8, Cell Counting Kit-8; GSEA, Gene Set Enrichment Analysis; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins; ECM, extracellular matrix.

multivariate Cox regression analysis when considering tumor size and T stage as independent prognostic factors. Therefore, it is imperative to assess tumor size as a prognostic factor through univariate Cox regression analysis. Previous studies have demonstrated that the macroscopic type affects the prognosis of patients with ICC (66). However, the small sample size of the present cohort made it impossible to evaluate the macroscopic type in the present study. The inclusion of additional observed variables necessitates a larger sample size; otherwise, the statistical tests may not meet the necessary requirements, leading to compromised repeatability and representativeness. This could potentially result in erroneous conclusions, including false negatives or false positives. Considering the purpose of the present study and the small sample size, the variables presented in Table II were selected. Multi-center studies on hepatobiliary clinic should be conducted in the future to examine other factors. Thirdly, no additional cell lines verified the role of ITGB5 in ICC, and no further experiments explored the signaling pathways associated with ITGB5 in ICC. In future studies on ITGB5-related signaling pathways and additional ICC cell lines (HuH28; RBE; SSP25) should be employed to confirm the reproducibility of the present findings.

For simplicity and clarity, a flowchart of the present study has been presented in Fig. 10. In conclusion, ITGB5 may act as a regulator of ICC development and progression by influencing the proliferation and invasion of ICC cells. ITGB5 could be a potential biomarker for a poorer prognosis of ICC in a Chinese population, and it may be helpful to screen candidates for receiving intensive therapy. However, future studies with large sample sizes are required to validate the role of ITGB5 in the prognosis of patients with ICC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LM and JZ designed the study, performed the experiments, analyzed the data and wrote the manuscript. LM and KS collected the data. LM and JZ confirm the authenticity of all the raw data. All authors agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work (including the provided data) are appropriately investigated and resolved. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Written informed consent was received from each patient at the time of surgery for the use of their tissues in research. The present study was conducted in accordance with the ethical standards defined in the Declaration of Helsinki and was approved by the Medical Ethics Committee of Taizhou People's Hospital (approval no. KY 2020-091-01).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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