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TIPE2 serves as a favorable prognostic biomarker and suppresses cholangiocarcinoma progression by targeting RAC1-mediated integrin αvβ6 trafficking

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Abstract

Background: Tumor necrosis factor-α-induced protein-8 (TNFAIP8/TIPE) like-2 (TIPE2), a critical member of the TIPE protein family, has been characterized as a tumor suppressor across multiple malignancies. Its functional significance in cholangiocarcinoma pathogenesis remains poorly defined.

Methods: To address this knowledge gap, we established a discovery cohort of 218 cholangiocarcinoma patients and an independent validation cohort of 95 cases. The expression of TIPE2 was explored via immunohistochemistry (IHC). The correlation between patients' clinical parameters including overall survival and TIPE2 expression were evaluated. A nomogram including TIPE2 expression was also constructed and validated to for prognostic prediction. The effect and related mechanisms of TIPE2 on the malignant behaviors of cholangiocarcinoma was investigated both *in vitro*, *in silico*, and *in vivo*.

Results: The two cohorts demonstrated that TIPE2 expression was decreased in cholangiocarcinoma tissues, and TIPE2 expression was closely related with tumor stage and the prognosis of cholangiocarcinoma patients. Nomogram including TIPE2 expression could predict the prognosis of cholangiocarcinoma patients effectively. TIPE2 suppressed the proliferation, migration and invasion capacities of cholangiocarcinoma cells, and inhibited tumor growth *in vivo*. Mechanistically, TIPE2 suppressed the trafficking of integrin αvβ6 via targeting RAC1, which subsequently suppressed the progression of cholangiocarcinoma.

Conclusion: Through comprehensive clinical cohorts and functional investigations, the present study identified TIPE2 as a clinically significant prognostic biomarker and revealed its potential role in regulating integrin-mediated oncogenic processes in

cholangiocarcinoma. Therapeutic enhancement of TIPE2 expression emerges as a promising precision medicine strategy for cholangiocarcinoma management.

Key Words: Cholangiocarcinoma, TIPE2, biomarker, integrin αvβ6, RAC1

Introduction

Cholangiocarcinoma is a neoplasm originating from the ductal epithelium of the biliary tree and is typically classified into intrahepatic cholangiocarcinoma (iCCA), extrahepatic cholangiocarcinoma (eCCA), and hilar cholangiocarcinoma (hCCA). It represents the most rare but aggressive of all biliary tumors [1]. Cholangiocarcinoma was initially described by Durand Fardel in 1840. The incidence and mortality of this rare disease is increasing over the last decades [2,3]. Several risk factors have been described, including advanced age, obesity, viral hepatitis (with or without cirrhosis), primary sclerosing cholangitis (PSC), chronic choledocholithiasis, bile duct adenoma, and parasitic biliary infestation [1,4,5]. Most patients with cholangiocarcinoma exhibit no specific symptoms in the early stages, leading to delayed diagnosis and a poor prognosis. Surgery is the only potentially curative treatment strategy, but only 25% of patients with cholangiocarcinoma are eligible for surgical intervention, which is often associated with high morbidity and mortality. Systemic chemotherapy is widely used for patients with disseminated or metastatic disease. Nevertheless, the 5-year overall survival rate is less than 5%, which has not significantly changed in the past 30 years [6]. Therefore, discovering novel biomarker for the risk stratification and prognostic prediction, along with a better understanding of the molecular mechanisms that are associated with the development of cholangiocarcinoma, is essential for improving the prognosis of patients with this devastating disease [4,7,8].

TIPE2 belongs to the TNFAIP8 family. The family consists of four members, TNFAIP8, TIPE1, TIPE2 and TIPE3, and all these four proteins consist of a death effector domain (DED) [9]. TIPE2 was initially discovered as an immune negative regulator and plays a vital

role in regulating immune homeostasis. It was primarily isolated from the inflamed spinal cord of experimental autoimmune encephalomyelitis (EAE) mice [10,11]. As an immune regulator, TIPE2 is preferentially expressed in lymphoid tissues and cells, and its deficiency usually leads to lethal systemic inflammation, showing hyper-sensitive to Toll-like receptor (TLR) and T cell receptor (TCR) stimulation. It has been reported that it is downregulated in patients with infectious or autoimmune diseases [10-12]. Moreover, recent research has revealed that aberrant TIPE2 expression occurs in several kinds of tumors, including renal cell carcinoma (RCC), non-small-cell lung cancer (NSCLC), rectal cancer, gastric cancer, bladder cancers *et al*. The expression patterns and effects of TIPE2 in different tumors varies a lot [13-16]. Of note, detailed mechanisms of the effect of TIPE2 were limited in most of these studies. In our previous research, we found that TIPE2 serves as a tumor suppressor in NSCLC and hepatocellular carcinoma (HCC), mainly functions via binding with RAC1 directly [17,18].

TIPE2 could significantly suppress the invasiveness and metastasis of tumor cells. Cell adhesion to the extracellular matrix is fundamental to tumor invasiveness and metastasis. While integrins belong to a family of cell surface adhesion molecules, which composed of α and β subunits that are non-covalently connected [19]. Integrins are implicated in nearly every step of cancer progression from primary tumor development to metastasis [20]. Integrin αvβ6 is the only heterodimer that the β6 subunit could form, and it is highly expressed during embryogenesis, tissue repair and carcinogenesis. Our previous research demonstrated that integrin αvβ6 is a key regulator during tumor progression in cholangiocarcinoma [21,22].

The expression and roles of TIPE2 in cholangiocarcinoma have not been reported so far. In the present study, we explored the

expression and clinical significance of TIPE2 in cholangiocarcinoma using both retrospective and prospective cohorts. We further investigated the role and corresponding mechanisms of TIPE2 in cholangiocarcinoma. Based on the internalization and recycling of integrin αvβ6, we explored the functions of TIPE2 from a new perspective. The present study demonstrated that TIPE2 may serve as a potential prognostic biomarker and a therapeutic target for this rare but devastating disease.

Patients and methods

Retrospective cohort and database

The retrospective cohort consisted of 218 patients diagnosed as cholangiocarcinoma that underwent radical surgical resections between 2009 and 2012 at the Department of General Surgery, Qilu Hospital of Shandong University. Clinicopathological classification and staging were determined according to the American Joint Committee on Cancer (AJCC) criteria. The inclusion criteria were as follows: (1) formalin-fixed tumor tissues and adjacent normal tissues with detailed clinicopathological data; (2) no adjuvant chemotherapy or radiotherapy; (3) postoperative survival time more than 1 month; (4) no history or signs of other malignancies. Follow-up data were recorded until December 2017, concerning survival time and progression of cholangiocarcinoma at the last visit. This study was approved by the Ethics Committee of Shandong University, and written informed consent was obtained from all the subjects.

Validation cohort

A validation cohort consisted of 95 cholangiocarcinoma patients was established between January 2016 to December 2018 at the Department of General Surgery, Qilu Hospital of Shandong University. Tumor tissues and corresponding adjacent normal

tissues were obtained. None of the patients received chemotherapy or radiotherapy prior to surgery. Detailed clinicopathological data (including serum CA19-9 levels) were recorded. Written informed consent was obtained from each patient. This study was approved by the Ethics Committee of Shandong University (KYLL-2016-226) and all methods were performed in accordance with the relevant guidelines and regulation. The procedures have been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments.

Immunohistochemistry (IHC)

IHC was performed using paraffin-embedded tissue sections (4 μm). The sections were dewaxed and hydrated, followed by antigen retrieval (in 0.01 mol/L citrate buffer solution, pH 6.0, heated to boiling for 2-3 min). Endogenous peroxidase was blocked with 3% hydrogen peroxide solution. The sections were incubated with the blocking goat serum for 15 min and then immunostained with rabbit antibody against TIPE2 (dilution 1:300, BOSTER, China) at 4℃ overnight. Secondary staining was performed with HRP-conjugated anti-rabbit IgG using a MaxVision Kit and a 3, 5-diaminobenzidine (DAB) peroxidase substrate kit (Maixin Co, Fuzhou, China). The sections were then counterstained with hematoxylin, and representative images were obtained under an Olympus inverted microscope.

Evaluation of immunohistochemical staining

Immunohistochemical staining was independently assessed by two experienced pathologists in a blinded manner. Staining was semi-quantitatively scored based on both the staining intensity (0, negative; 1, very weak; 2, weak; 3, moderate; 4, strong) and the percentage of positively stained cells (0, 0%; 1, 1%-25%; 2, 26%-50%; 3, 51%-75%; 4, 76%-100%). Then the two scores for each specimen

were combined to obtain the final score of TIPE2 expression (ranging 0-8). The cut-off point for the sum of the scores was defined as follows: 0-3, low expression; 3-8, high expression, which was verified by the X-tile program.

Nomogram Construction and Validation

The categorical variables were compared using the χ2 or Fisher's exact test. The Kaplan-Meier method was used for univariate survival analysis. Meaningful predictive factors were screened out according to the standard of $P < 0.05$ and Cox's proportional hazards regression model was used for multivariate analysis. Nomogram was built that included description of independent prognostic factors. Besides, the prognosis of nomogram for first, third, and fifth year was realized using the RMS software package in R software version3.5.0 (https://www.r-project.org/). Validation of the nomogram was performed using concordance statistics. The study used the concordance index (C-index) and 95% confidence interval (CI) to determine the discrimination ability. The calibration curves were used to compare the predicted results of the nomogram with the actual results, while the 45-degree reference line was used as the optimal model. Furthermore, decision curve analysis (DCA) could be used as a novel algorithm for evaluating the clinical efficacy of the model, which has some advantages over AUC.

Cell culture

Human cholangiocarcinoma cell lines including RBE and QBC939 were purchased from the American Type Culture Collection (ATCC) and were separately cultured in RPMI 1640 and DMEM medium supplemented with 10% inactivated fetal bovine serum (FBS) (Gibco, CA, USA) in a humidified cell incubator with an atmosphere of 5% CO2 at 37℃.

Plasmid construction and transfection

The TIPE2 plasmid was generated from the cDNA clone by PCR and cloned in frame with a C-terminal Flag into vector PRK5. The mutant TIPE2 in which the TIPE2 N-terminal lysine or arginine residues, Lys-15, Lys-16, and Arg-24 were replaced with glutamine or alanine was generated by PCR-based site-directed mutagenesis. The TIPE2-shRNA sequences purchased from GenePharma were as follows: forward 5'-GCACAUUCCACCUUGACAATT-3'; reverse 5'-UUGUCAAGGUGGAAUGUGCTT-3'. The negative control shRNA sequence: forward 5'-UUCUCCGAACGUGCUACGUTT-3', reverse 5'-ACGUGACACGUUCGGAGAATT-3'. Transfection of cholangiocarcinoma cells with plasmid or shRNA was performed using X-tremeGENE transfection reagent according to the manufacturer's protocols (Roche, Switzerland).

RNA isolation and quantitative real-time PCR

Total RNA was extracted from transfected cholangiocarcinoma cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and were reverse-transcribed into cDNA using a ReverTra Ace qPCR Kit (Toyobo, Osaka, Japan). Quantitative real-time PCR (qPCR) was performed using an Ultra SYBR Mixture (CWBIO). The primers used in this study are listed in Supplementary Table 1.

Cell proliferation assay

Cholangiocarcinoma cells (3000/well) were seeded in 96-well plates in triplicate wells and cultured for indicated time points. Cell proliferation was evaluated using the Cell Counting Kit-8 (CCK8) (Beyotime, Haimen, China) assay according to the manufacturer's instructions. The absorbance was determined at 450 nm.

Trans-well assays for cell migration and invasion

The migration and invasion capacities of cholangiocarcinoma cells were analyzed in 24-well Boyden chambers with 8-μm pore size polycarbonate membranes (Costar, Acton, USA). For invasion assay,

the membranes were pre-coated with 50 μg Matrigel (BD Biosciences, San Diego, USA) to simulate matrix barriers. Cells (5 × 10^5/ml) were resuspended in 200 μl serum-free medium and placed in the upper chamber. The lower compartments were filled with 600 μl medium with 10% FBS. The cells left on the upper surface of the membrane were removed after incubation for certain times. While the cells on the lower surface of the membrane were fixed with methanol for 10 minutes and then stained with crystal violet for 20 minutes. Stained cell counting was performed under a light microscope at ×200 magnification.

Subcutaneous xenograft mouse model

BALB/C nude mice (4-6 weeks old) were obtained from the Chinese Academy of Sciences (Shanghai, China) and maintained in laminar-flow cabinets under specific pathogen-free conditions (SPF). Cholangiocarcinoma cell line RBE was used to establish the orthotopic xenograft tumor model. Cells were harvested by trypsinization and were resuspended in the culture medium. A total number of $1*10^7$ cells in a volume of 50 μl were injected subcutaneously into the flanks of nude mice. Ten days after subcutaneous inoculation, mice were divided randomly into two groups and treated by intratumoral injection of TIPE2 plasmid or MOCK plasmid complexed with transfection reagent *in vivo* jet PEI (Polyplus-transfection Inc., New York, USA). The intratumoral injection was conducted every other day for 12 days. All the mice were sacrificed by cervical dislocation after 5-6 weeks and tumors were separated and weighed, then processed for histopathological examination. All the animal experiments were conducted according to the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Scientific Investigation Board of Qingdao University (Qingdao, Shandong Province, China).

All the procedures of the study were carried out in accordance with the ARRIVE guidelines.

Internalization assay and recycling assay

The internalization assay was conducted as previous reported [23]. Briefly, cholangiocarcinoma cells were labeled with 0.2 mg/ml EZ-Link Sulf-NHS-SS-Biotin at 4℃ for 1 h. Labeled biotin was removed from proteins remaining at the cell surface by treatment with a solution containing 20 mM MesNa (a non-cell-permeant reducing agent), 50 mM Tris at pH 8.6, and 100 mM NaCl for 15 min at 4℃. MesNa was quenched by using 20 mM IAA for 10 min at 4℃. Then cells were lysed, and the amount of biotinylated integrin αvβ6 was determined by ELISA. For recycling assay, cholangiocarcinoma cells were transferred to DMEM for 10min (early internalization) or 30min (late internalization) at 37℃ following surface labeling with 0.2 mg/ml EZ-Link Sulf-NHS-SS-Biotin for 60 min at 4℃. Then biotin labeled cells were incubated in MesNa solution to remove biotin on the cell surface. Then cells were quenched with 20m MIAA after indicated times, and the levels of biotinylated integrin αvβ6 were determined by ELISA. The integrin αvβ6% recycled back to the plasma membrane was defined as the percentage of the integrin pool labeled during the internalization period.

Molecular docking procedure

Molecular docking was performed to investigate the binding interactions among the human TIPE2, the human RAC1, and the human integrin β6 using the ZDOCK server (http://zdock.umassmed.edu/). The three-dimensional (3D) structures of TIPE2 (PDB ID: 3F4M), the RAC1 (PDB ID: 3TH5), and integrin β6 (PDB ID: 4UM8) were obtained from the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). For docking, the default parameters specified on the ZDOCK server were utilized. The

highest-ranked pose, as determined by the docking score, was subjected to visual analysis using PyMoL version 1.7.6 software (http://www.pymol.org/).

Statistical analysis

The associations between TIPE2 expression and clinicopathological parameters were analyzed by the χ^2 test or Fisher's exact test. The cumulative OS rates were calculated by Kaplan–Meier method, and the statistical differences between subgroups were calculated by log-rank test. Independent prognostic factors were identified by multivariate analysis with Cox-regression model. The statistical comparisons between control and tested group were analyzed with the one-way, two-way ANOVA or t tests. All statistical analyses were performed using SPSS 18.0 software (SPSS Inc., Chicago, USA), and a P value < 0.05 was considered statistically significant.

Results

TIPE2 expression is down-regulated in cholangiocarcinoma and associated with tumor stage.

To explicit the expression of TIPE2 in cholangiocarcinoma, IHC was conducted using a cohort that consist of 218 patients. Results showed that TIPE2 mainly localized in the cytoplasm of tumor cells, and TIPE2 expression was decreased in cholangiocarcinoma tissues compared with corresponding adjacent normal tissues (Figure 1A-B). TIPE2 expression was gradually decreased with increased tumor stage (Figure 1C-E).

The association between TIPE2 expression and related clinicopathological parameters were analyzed, and results showed that TIPE2 expression was closely associated with tumor size, T stage, N stage, TNM stage and vascular invasion (Table 1). Then the

receiver operator characteristic (ROC) curves were constructed to evaluate the clinicopathological significance of TIPE2 in cholangiocarcinoma. Results revealed that the area under the curve (AUC) for the diagnosis of cholangiocarcinoma, prediction of lymph node metastasis and prediction of TNM stage was up to 0.752 (P<0.001), 0.604 (P=0.040) and 0.699 (P<0.001), respectively (Figure 1F-H).

TIPE2 is a potential favorable tumor biomarker in cholangiocarcinoma.

Univariate analysis to investigate the prognostic value of TIPE2 expression in the overall survival (OS) of cholangiocarcinoma patients. In this cohort, cholangiocarcinoma patients with large tumor size (P<0.001), advanced N stage (P<0.001) /TNM stage (P<0.001), poorly differentiation (P=0.020), vascular invasion (P<0.001) and low TIPE2 expression (P<0.001) tended to have poor prognosis (Supplementary Table 2, Figure 1I).

Multivariate analysis was further conducted to reveal the independent prognostic factors. Factors with P value <0.1 in the previous univariate analysis were involved into the Cox-regression model for multivariate analysis, which showed that large tumor size (P=0.009), N stage (P=0.014), vascular invasion (P=0.039) and low TIPE2 expression (P<0.001) were independent unfavorable prognostic factors (Supplementary Table 2).

Construction and validation of the nomogram based on TIPE2 expression.

Based on the independent prognostic analysis, nomogram was constructed to evaluate the predictive ability of the 1-year, 3-year and 5-year OS in patients with cholangiocarcinoma (Figure 2A). In the nomogram, the following scoring criteria were applied: a tumor size of ≤3 cm was assigned 0 points, while a size >3 cm was assigned

61 points; for vascular invasion, the absence of invasion was assigned 0 points, and its presence was assigned 52 points; for the N stage, N0 was assigned 0 points and N1 59 points; for TIPE2 expression, high expression was assigned 0 points, while low expression was assigned 100 points. Essentially, for an excellent visualization tool, each factor in the nomogram was given different values according to the regression coefficient, the sum of which represents the total score. A greater value indicated poorer patient survival and a higher mortality risk. For nomogram validation, the performance of the model was quantified concerning the discrimination and calibration. The C-index of the model was 0.68 (95% confidence interval: 0.634-0.726), indicating that it had a good discriminating ability. Furthermore, the obtained calibration curve suggested good calibration of the new model in predicting the survival of patients for certain years (Figure 2B-D). The last component of the nomogram performance assessment was clinical usefulness. DCA showed that the established nomogram displayed good net clinical benefit compared with the traditional TNM staging system (Figure 2E).

Clinical significance of TIPE2 expression in the validation cohort.

Our previous results found that TIPE2 expression was associated with the tumor stage and the prognosis of cholangiocarcinoma patients. To certify our conclusion, we further explored the correlation between TIPE2 expression and clinicopathological parameters of cholangiocarcinoma patients in a validation cohort consisting of 95 patients (Supplementary Table 3). TIPE2 expression was also decreased in tumor tissues compared with adjacent normal tissues (Supplementary Figure 1A). TIPE2 expression was even lower in tumor tissues with lymph node metastasis (Supplementary

Figure 1B), and TIPE2 expression gradually decreased accompanied with advanced tumor stage (Supplementary Figure 1C-D). Moreover, we found that TIPE2 expression was closely associated with serum CA19-9 of cholangiocarcinoma patients in this cohort (Supplementary Figure 1E). These results convinced that TIPE2 was a potential IHC biomarker for tumor malignancy.

TIPE2 suppresses the proliferation, migration and invasion capacities of cholangiocarcinoma.

Our precedent findings demonstrated that TIPE2 might play a pivotal role in the progression of cholangiocarcinoma. Therefore, we then explored the effect of TIPE2 on the malignant behaviors of cholangiocarcinoma both *in vitro* and *in vivo*. Human cholangiocarcinoma cell lines RBE and QBC939 were used in the subsequent research. TIPE2 shRNA and overexpression plasmid were constructed and transfected, the knockdown and overexpression efficiency were confirmed (Figure 3A). The effect of TIPE2 on the proliferation of cholangiocarcinoma cells was assessed by CCK-8 assay, and results showed that the proliferation capacity of cholangiocarcinoma cells was markedly enhanced after TIPE2 silencing, while overexpression of TIPE2 suppressed the proliferation of cholangiocarcinoma cells (Figure 3B-C). Trans-well migration and invasion assays were conducted, and results showed that the migration and invasion capacities were increased after knockdown of TIPE2, while TIPE2 overexpression suppressed the migration and invasion of RBE and QBC939 cells (Figure 3D-G).

To investigate the effect of TIPE2 *in vivo*, a subcutaneous xenograft tumor model in nude mice bearing RBE cells was established. Results revealed that TIPE2 treatment significantly suppressed the growth of tumors, tumors in the TIPE2 treatment group had a relatively smaller volume and weight (Figure 3H-K). All

these results indicated that TIPE2 suppressed tumor progression both *in vitro* and *in vivo*.

The trafficking of integrin αvβ6 is involved in the progression of cholangiocarcinoma.

It is well known that the integrin family are involved in almost every step of cancer progression. While our previous research demonstrated that integrin αvβ6 was up-regulated and promoted tumor progression in cholangiocarcinoma. To investigate the detailed mechanism for the effect of TIPE2 on tumor progression, we conducted the subsequent experiments. Firstly, using internalization assays and recycling assays, we confirmed that integrin αvβ6 is constitutively internalized into the cytoplasm and recycled to the cell membrane in cholangiocarcinoma cells (Figure 4A-B). Then the internalization inhibitor Bolinaquinone (BLQ) was used, and results showed that BLQ treatment suppressed the internalization but not the recycling of integrin αvβ6 (Figure 4C-D).

Moreover, CCK-8 and Trans-well assays were conducted after BLQ treatment in cholangiocarcinoma cells, and the results demonstrated that after inhibiting of internalization of integrin αvβ6, the proliferation, migration and invasion capacities were significantly suppressed (Figure 4E-F). All these results indicated that the trafficking of integrin αvβ6 was involved in the progression of cholangiocarcinoma.

RAC1 was involved in the process of integrin αvβ6 trafficking.

Our previous results found that TIPE2 could suppress the malignant behaviors of cholangiocarcinoma cells, and the trafficking of integrin αvβ6 is involved in the progression of cholangiocarcinoma. To verify whether TIPE2 functions through regulating αvβ6 trafficking, we designed the following experiments. First of all, the internalization assay and recycling assay were conducted after

TIPE2 transfection in cholangiocarcinoma cells, and results showed that TIPE2 suppressed the internalization and recycling significantly (Figure 5A-B).

As our previous research revealed that TIPE2 binds with RAC1 directly and suppresses RAC1 activity, while RAC1 is essential for the process of internalization and recycling of cell membrane molecules. Then NSC23766, a specific RAC1 activity inhibitor was used in the subsequent experiments. Accordingly, the treatment of NSC23766 in cholangiocarcinoma cells could markedly suppress the internalization and recycling of integrin αvβ6 (Figure 5C-D), suggesting that RAC1 was a key regulator of integrin αvβ6 trafficking. These results indicated that TIPE2 may suppresses the trafficking of integrin αvβ6 via targeting RAC1.

TIPE2 suppressed the trafficking of integrin αvβ6 via targeting RAC1.

As our aforementioned results indicated that RAC1 was involved in the trafficking of integrin αvβ6, while our previous research demonstrated that TIPE2 suppresses tumor progression via binding with RAC1. We hypothesized that TIPE2 regulates integrin αvβ6 trafficking via RAC1 in CCA. To confirm our hypothesis, we performed computational molecular docking and dynamics simulations to provide direct evidence for the TIPE2-Rac1-integrin αvβ6 axis. The interaction between the integrin β6 (cyan) and the RAC1 (green) was shown in Figure 6A. Detailed analysis showed that one hydrophobic interaction was observed between the residues Leu-371, Phe-373 of the integrin β6 and the residues Ala-27, Phe-28 and Leu-160 of the RAC1, while another hydrophobic interaction was observed between the residues Phe-259, Met-261, Ile-295, Ile-299, Leu-320, Tyr-324 and Leu-327 of the integrin β6 and the residues Val-36, Phe-37, Tyr-40, Trp-56, Tyr-64, Leu-67 and Leu-70 of the

RAC1, forming strong hydrophobic bindings (Figure 6B). In addition, the residue Glu-367 of the integrin β6 formed an anion-π interaction with the Tyr-23 of the RAC1. Importantly, six hydrogen bond interactions were shown between the residue Gly-368 of the integrin β6 and the Lys-166 of the RAC1 (bond length: 2.7 Å), the Thr-366 and Leu-369 of the integrin β6 and the Asn-26 of the RAC1 (bond length: 2.2 and 2.2 Å), the Lys-387 of the integrin β6 and the Phe-28 of the RAC1 (bond length: 2.6 Å), the Asn-323 of the integrin β6 and the Thr-35 of the RAC1 (bond length: 3.3 Å), the Ile-295 of the integrin β6 and the Phe-37 of the RAC1 (bond length: 2.0 Å), which were the main binding affinity between the integrin β6 and the RAC1.

The interaction between the TIPE2 (rose red) and the integrin β6 (cyan) was shown in Figure 6C. Detailed analysis showed that one hydrophobic interaction was observed between the residues Ile-31 and Ile-64 of the TIPE2 and the residues Met-261 and Pro-271 of the integrin β6, while another hydrophobic interaction was observed between the residues Ile-68, Val-72, Leu-73 and Leu-135 of the TIPE2 and the residues Phe-259, Ile-295, Ile-299, Leu-320, Tyr-324 and Ile-328 of the integrin β6, forming strong hydrophobic bindings (Figure 6D). In addition, the residues Lys-65 and Lys-69 of the TIPE2 formed cation-π interactions with the residues Phe-259 and Tyr-324 of the integrin β6. Importantly, seven hydrogen bond interactions were shown between the residues His-133 and Ser-78 of the TIPE2 and the Lys-326 of the integrin β6 (bond length: 2.8 and 3.1 Å), the Arg-75 of the TIPE2 and the Gly-296 of the integrin β6 (bond length: 2.7 and 3.4 Å), the Lys-69 of the TIPE2 and the Tyr-324 of the integrin β6 (bond length: 1.9 Å), the Lys-65 of the TIPE2 and the Asp-262 of the integrin β6 (bond length: 2.8 Å), the Asp-40 of the TIPE2 and the Lys-264 of the integrin β6 (bond length: 3.4 Å), which were the main binding affinity between the TIPE2 and the integrin β6. In

addition, the estimated ZDOCK scores were 1270 for the integrin β6 and the RAC1, and 1347 for the TIPE2 and the integrin β6, respectively.

To further confirm our conclusion, a TIPE2-Mutant plasmid was used in the subsequent rescue experiments. The TIPE2-Mutant plasmid had deficiency in binding with RAC1, which intervened the TIPE2-RAC1 interaction. Correspondingly, we found that the effect of TIPE2 on the proliferation, migration and invasion of cholangiocarcinoma cells vanished after losing the TIPE2-RAC1 interaction (Figure 5E-G), indicating that TIPE2 functions through targeting with RAC1 in cholangiocarcinoma. Furthermore, the effect of TIPE2 was also diminished after pre-treatment of NSC23766 in cholangiocarcinoma cells (Figure 5H-J). All these results demonstrated that TIPE2 suppressed tumor progression via targeting RAC1, which subsequently interfered the trafficking of integrin αvβ6 in cholangiocarcinoma.

Discussion

The overall survival for cholangiocarcinoma patients has not significantly changed in the past decades. Discovering effective biomarker is of vital importance for the precise risk stratification and the development of targeted therapies for cholangiocarcinoma patients, which is urgently needed for improving the prognosis of this devastating disease [7,24,25]. In the present study, we demonstrated that TIPE2 serves as a favorable prognostic biomarker in cholangiocarcinoma and may contribute to risk stratification. Of note, TIPE2 markedly suppressed the progression of cholangiocarcinoma both *in vitro* and *in vivo*. Mechanistically, TIPE2 inhibited the trafficking of integrin αvβ6 via targeting RAC1, which subsequently suppressed the malignant behaviors of

cholangiocarcinoma cells. The key novelty of the present study lies in: (1) Demonstration of TIPE2 downregulation and its prognostic significance in CCA. (2) Integration of TIPE2 into a clinically applicable nomogram for CCA prognosis prediction, which is important for clinical decision making and prognostic prediction. (3) Identification of the TIPE2-RAC1-αvβ6 trafficking axis in CCA.

TIPE2 was initially identified as a negative regulator of the immune system and plays a pivotal role in regulating immune homeostasis. Most research on TIPE2 has focused on immune-related diseases [10,12,26,27]. But the expression pattern of TIPE2 indicates that it may functions beyond immune regulation, as it is commonly expressed in glandular epithelium, and dysregulated TIPE2 expression may contribute to tumorigenesis and tumor progression [28-30]. Previous research has revealed the expression and roles of TIPE2 in common cancer such as renal cell cancer, non-small-cell lung cancer, hepatocellular carcinoma and gastric cancer [14-16,31]. However, the expression and functions of TIPE2 in cholangiocarcinoma, a rare but fetal tumor, has not been reported till now. Cholangiocarcinoma derives from the glandular epithelium of the bile duct, and it is traditionally classified into intrahepatic cholangiocarcinoma, perihilar cholangiocarcinoma and distal cholangiocarcinoma [32]. As TIPE2 is selectively expressed in glandular epithelium with secretory function [29], we highly suspect that TIPE2 expression may also dysregulated during the tumorigenesis. In the present study, we constructed a cohort consists of 218 patients and explored the expression of TIPE2 in cholangiocarcinoma. We found that TIPE2 was down-regulated in cholangiocarcinoma tissues compared with the adjacent normal tissues, and TIPE2 expression was significantly associated with several clinicopathological parameters such as tumor size, TNM

stage and vascular invasion. Moreover, high TIPE2 expression served as an independent favorable prognostic factor for cholangiocarcinoma patients. These results indicated that TIPE2 was a potential biomarker for risk stratification and might be involved in the progression of cholangiocarcinoma.

The key reason for the poor prognosis of cholangiocarcinoma patients is lack of effective tumor biomarkers that can be used for early diagnosis and risk stratification [33-35]. According to the results of the multivariate Cox regression analysis, tumor size, vascular invasion, N stage, and TIPE2 expression were identified as prognostic indicators. The TNM staging system remains the gold standard for assessing tumor prognosis; however, it does not account for the impact of genetic alterations. Tumor size and vascular invasion were associated with the duration of tumor presence, local invasion, and distant metastasis, resulting in a poor prognosis for patients. Based on these factors, we developed a multivariate clinical prediction model. Using this modeling approach, we have identified TIPE2 expression as one of the most critical factors influencing survival in patients with cholangiocarcinoma. Compared with traditional TNM staging, the nomogram found that the survival of patients with cholangiocarcinoma was also influenced by tumor burden and tumor marker. The combination of these elements from the TNM staging system with other tumor-associated indices would surely contribute to a better discriminatory power and calibration of the nomogram in predicting survival. In DCA, nomogram was compared with the traditional staging system to demonstrate higher clinical utility and net benefit of the predictive model in supporting clinical decisions. Therefore, the establishment of prognostic nomogram model for predicting the survival of patients with cholangiocarcinoma would be of high clinical application value.

Moreover, to confirm our conclusion, we constructed another validation cohort consists of 95 patients. In accordance with our previous results, TIPE2 expression was decreased in cholangiocarcinoma tissues, and it's expression also associated with tumor TNM stage. Taken together, all these results revealed that TIPE2 serves as a potential biomarker for predicting the prognosis of cholangiocarcinoma patients and provided a theoretical basis for the development and progression of cholangiocarcinoma. Therefore, we recommend a routine TIPE2 IHC staining for pathological examination of cholangiocarcinoma tissues to evaluate the risk stratification and predict the prognosis of patients.

TIPE2 was primarily identified as a key regulator in immune homeostasis. Recently research of TIPE2 has been focused on tumorigenesis [36,37]. The expression of TIPE2 differs in different tumors, TIPE2 mainly presents a low-expression pattern in most of the tumors, such as breast cancer, cervical cancer, endometrial cancer, gastric cancer, glioma, HCC, NSCLC, ovarian cancer, *etc* [15,17,18,38-42]. But TIPE2 is relatively high expressed in colorectal cancer and renal cell cancer [43,44]. TIPE2 mainly acts as a tumor suppressor during tumorigenesis and tumor progression. Here, we investigated the role of TIPE2 in cholangiocarcinoma. Using both *in vitro* and *in vivo* experiments, we demonstrated that TIPE2 could suppress tumor progression of cholangiocarcinoma, indicating that TIPE2 may serve as a potential treatment strategy for cholangiocarcinoma.

Integrins play a role in nearly every stage of cancer progression and metastasis, and emerging strategies that target integrins and antagonize specific integrin subunits represent promising advances in precision cancer therapy [45]. Integrin αvβ6 is the only heterodimer that the β6 subunit can form, it is selectively expressed in epithelium-

derived tumors, and various of classic oncogenic signaling pathways are involved in integrin αvβ6-mediated tumor progression [21]. The expression pattern and vital role of integrin αvβ6 in the progression of tumors make it an ideal target for specific tumor imaging and precise therapy. It has been reported that the activation of PKC accelerates the internalization and recycling of integrin αvβ6, which leads to redistribution of integrin αvβ6 on the membrane of tumor cells . This process increases cell motility and is of great importance for the down-stream signaling of integrin αvβ6 [23,46]. Importantly, we revealed that integrin αvβ6 trafficking plays a key role during the progression of cholangiocarcinoma. Moreover, TIPE2 suppresses the malignant behaviors of cholangiocarcinoma cells via inhibiting αvβ6 trafficking. This study provides relatively novel insights into the function of TIPE2.

Most previous studies provided limited details regarding the mechanisms underlying the effect of TIPE2. In our previous research, we found that TIPE2 serves as a tumor suppressor in NSCLC and HCC, mainly functions via binding with RAC1 directly [13,18]. RAC1 is a member of Rho GTPase family, which has been demonstrated to be involved in the progression of various tumors. It has been found that RAC1 is a key regulator in promoting the progression of cholangiocarcinoma, and targeting RAC1 might be a potential therapeutic approach for the treatment of cholangiocarcinoma [22]. Moreover, RAC1 also contribute to the process of cytoskeletal reorganization and vesicular trafficking [47]. Therefore, we hypothesized that TIPE2 may suppress integrin αvβ6 trafficking by targeting RAC1. By computational molecular docking and dynamics simulations, predicted potential interactions between TIPE2 and the cytoplasmic domain of integrin β6, as well as integrin β6 and RAC1 were identified. Further rescue validations using TIPE2-Mutant

plasmid and a RAC1-specific inhibitor also demonstrated that RAC1 is of vital importance during the process of integrin αvβ6 trafficking, and that TIPE2 suppresses integrin αvβ6 trafficking by inhibiting RAC1, subsequently decreasing the proliferation, migration and invasion capacities of cholangiocarcinoma cells.

A highlight for the present study is the establishment of two cohorts of cholangiocarcinoma patients, and the sample size is relatively large for this rare tumor, which is of great clinical significance. Focused on the trafficking of integrin αvβ6, this study presented a potential mechanism of TIPE2 functions from a new perspective. We recommend routine immunohistochemical staining of TIPE2 in cholangiocarcinoma tissues to assess tumor aggressiveness and to identify patients eligible for targeted therapies against RAC1 or integrin αvβ6. Moreover, serum integrin expression may serve as a potential biomarker for tumor aggressiveness [48], further investigation into the clinical significance of specific serum integrin expression in cholangiocarcinoma is still required. As this is a retrospective cohort study, a relatively high censoring rate in the survival analysis contributes to the instability of the Kaplan-Meier estimated survival probabilities. Although the changes in the at-risk population and the censoring rate over time are clearly indicated in the survival curve, this remains a limitation of the present study. Therefore, a multi-center prospective cohort study is required to evaluate the clinical significance of TIPE2 as a biomarker for cholangiocarcinoma, as well as to validate of the TIPE2-incorporated nomogram. Moreover, the downregulation of TIPE2 in cholangiocarcinoma may be attributed to epigenetic modifications, such as promoter hypermethylation, or transcriptional repression by oncogenic signals. It has been reported that the 1331bp region upstream TIPE2 gene has high promoter

activity. In other cancers, TIPE2 has been shown to be suppressed by inflammatory cytokines or hypermethylation. Actually, the upstream regulation of TIPE2 expression in different disease is a hot issue that warrant further investigation [9,49].

Notably, although four members of the TIPE family exhibit high homology, their expression patterns and functions differ significantly. Therefore, further research is warranted to elucidate the underlying mechanisms of this phenomenon. The original TIPE family member, TNFAIP8, functions as a negative regulator of apoptosis and serves as an oncogenic factor in tumor progression. While TIPE1 could induce cell apoptosis and inhibit tumorigenesis. TIPE2 functions as a negative regulator of immunity and inflammation and also acts as a tumor suppressor in certain neoplastic diseases. The newly identified TIPE3 protein functions as the transfer protein of phosphoinositide second messengers and has the potential to promote cancer. Variations in the expression and function of different TIPE family members may result from subtle structural differences. For instance, TIPE3 possesses an additional N-terminal domain compared to other family members, and its function has undergone significant alterations. As previously described, the N-terminal lysine or arginine residues of TIPE2 are essential for its binding to RAC1. Furthermore, the functions of TIPE family members may be influenced by the regulation and intervention of various factors across different diseases and varying stages of the same disease. This is also one of the issues that warrants careful consideration in translational medicine research on TIPE2 as a potential target for tumor treatment.

In conclusion, the present study reveals a new potential biomarker for the risk stratification and prognostic prediction in cholangiocarcinoma. Moreover, this study provides a potential

mechanism by which TIPE2 functions during tumorigenesis. As the downstream effectors of TIPE2, both αvβ6 and RAC1 have been demonstrated to be key regulators in cancer progression, indicating that enhanced TIPE2 may serve as a promising treatment strategy for this devastating disease.

Conflicts of interest

There are no conflicts to declare.

Data availability statement

All the related data are available upon request by contacting with the corresponding author.

Author Contributions

ZL designed the study, acquired funding and edited the manuscript, SW and WJ performed the experiments, analyzed the results and wrote the original draft, YS and CM collected and examined the data, JN contributed to the establishment of two cohorts and reviewed the manuscript. All authors read and approved the final manuscript.

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Figures and figure legends

Figure 1

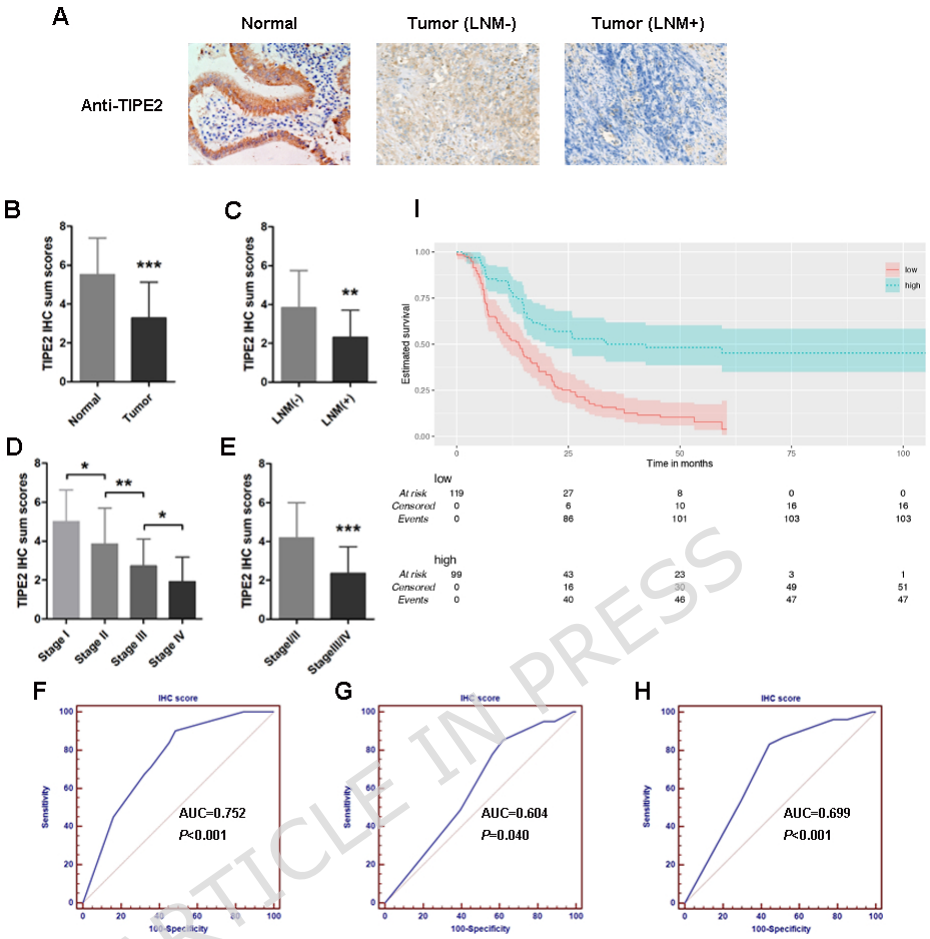

Figure 1. The expression and clinicopathological significance of TIPE2 in cholangiocarcinoma.

A. Representative IHC staining of TIPE2 in cholangiocarcinoma tissues and adjacent normal tissues (×200); LNM-: lymph node metastasis negative, LNM+: lymph node metastasis positive.

B. IHC sum scores were used to evaluate TIPE2 expression in cholangiocarcinoma tissues and adjacent normal tissues.

C. IHC sum scores were used to access TIPE2 expression in cholangiocarcinoma tissues with or without lymph node metastasis.

D-E. TIPE2 IHC sum scores in different stages of

cholangiocarcinoma.

F-H. ROC curves were established to evaluate the clinicopathological significance of TIPE2 in cholangiocarcinoma. (F) Tumor tissues v.s. normal tissues; (G) Tumor tissues without lymph node metastasis v.s. tumor tissues with lymph node metastasis; (H) Tumor tissues with different TNM stage.

I. Survival analysis according to TIPE2 expression in the cohort of patients with cholangiocarcinoma. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Figure 2

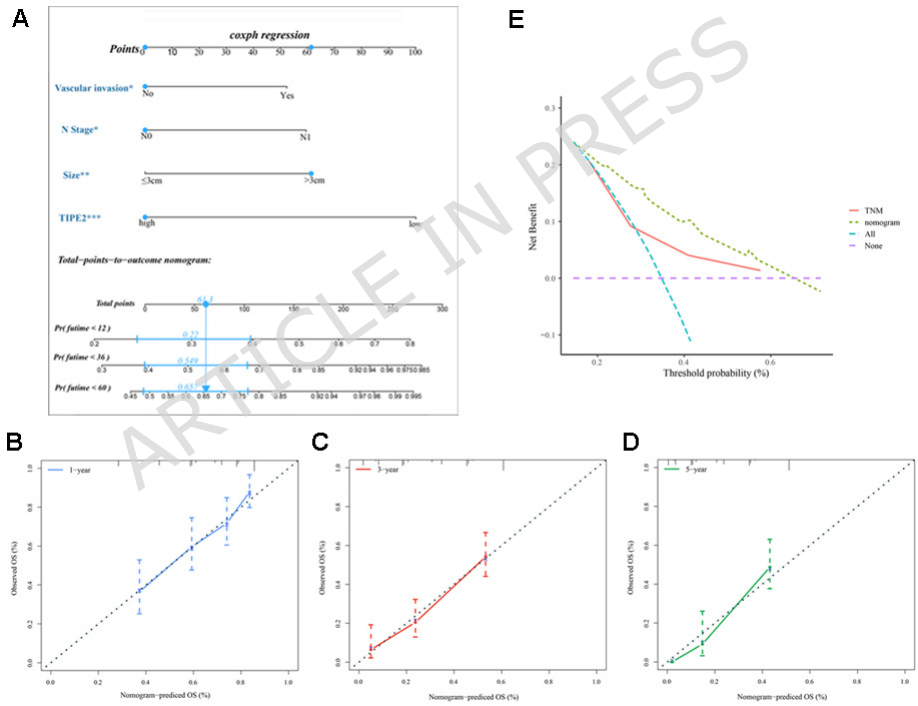

Figure 2. Establishment and verification of the nomogram.

A. Nomogram for predicting 1-, 3- and 5- overall survival of cholangiocarcinoma patients.

B-D. 1-, 3-, 5-year nomogram calibration curves of the prognostic nomogram.

E. Decision curve analyses of comparing nomogram with TNM stage.

Figure 3

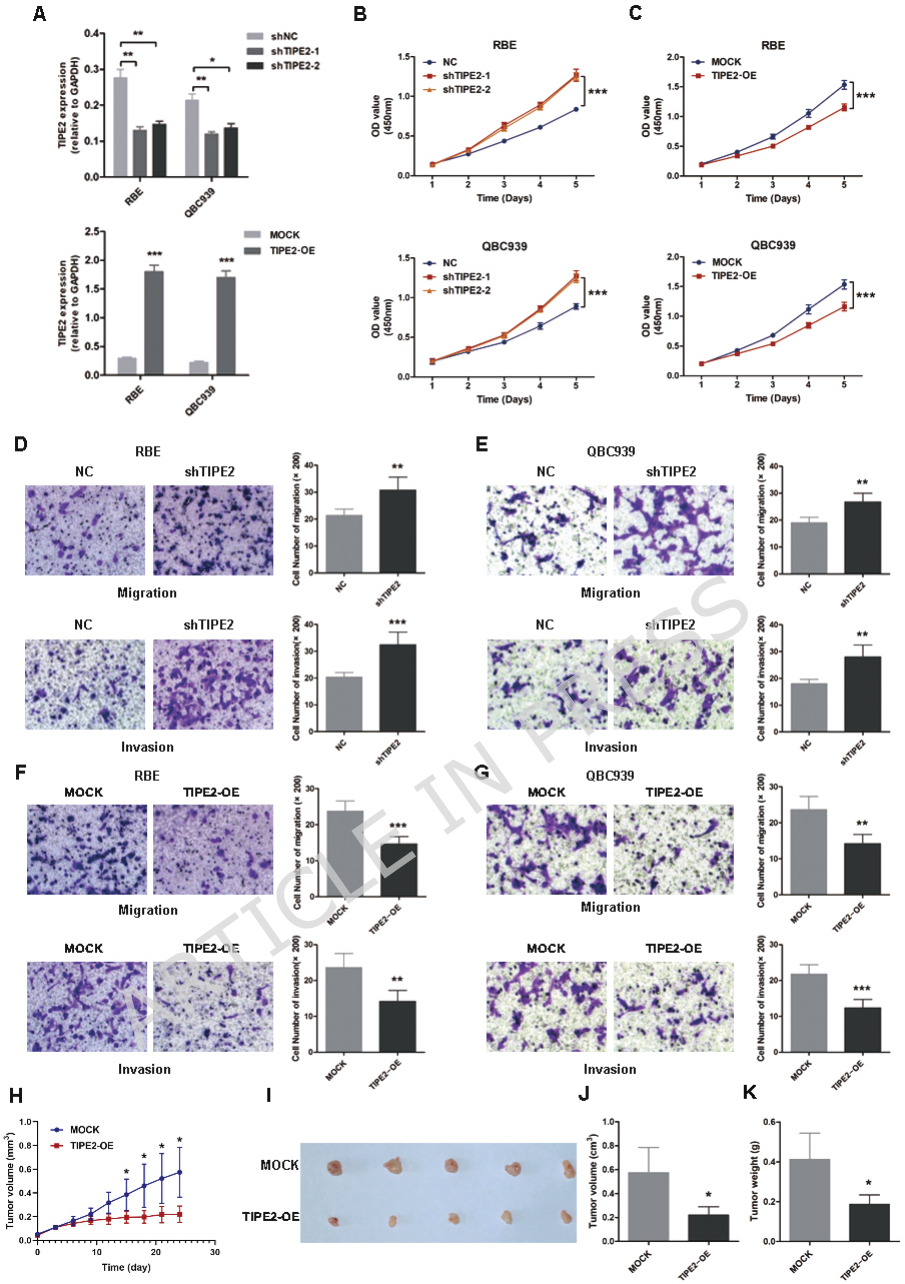

Figure 3. TIPE2 suppresses the malignant behaviors of cholangiocarcinoma both *in vitro* and *in vivo*.

A. The expression of TIPE2 were detected after TIPE2 silencing or overexpression using RT-PCR.

B. CCK-8 assays were performed following TIPE2 silencing in RBE and QBC939 cells.

C. CCK-8 assays were performed following TIPE2 overexpression in RBE and QBC939 cells.
D. Trans-well migration and invasion assays were conducted after TIPE2 silencing in RBE cells.
E. Trans-well migration and invasion assays were conducted after TIPE2 silencing in QBC393 cells.
F. Trans-well migration and invasion assays were conducted after TIPE2 overexpression in RBE cells.
G. Trans-well migration and invasion assays were conducted after TIPE2 overexpression in QBC939 cells.
H. Tumor growth curves in the subcutaneous xenograft model for the TIPE2-OE group and the MOCK group.
I. Representative pictures of xenograft subcutaneous tumors in the TIPE2-OE group and the MOCK group. (bar=1cm)
J. Tumor volumes in the TIPE2 group and the MOCK group.
K. Tumor weights in different groups. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Figure 4

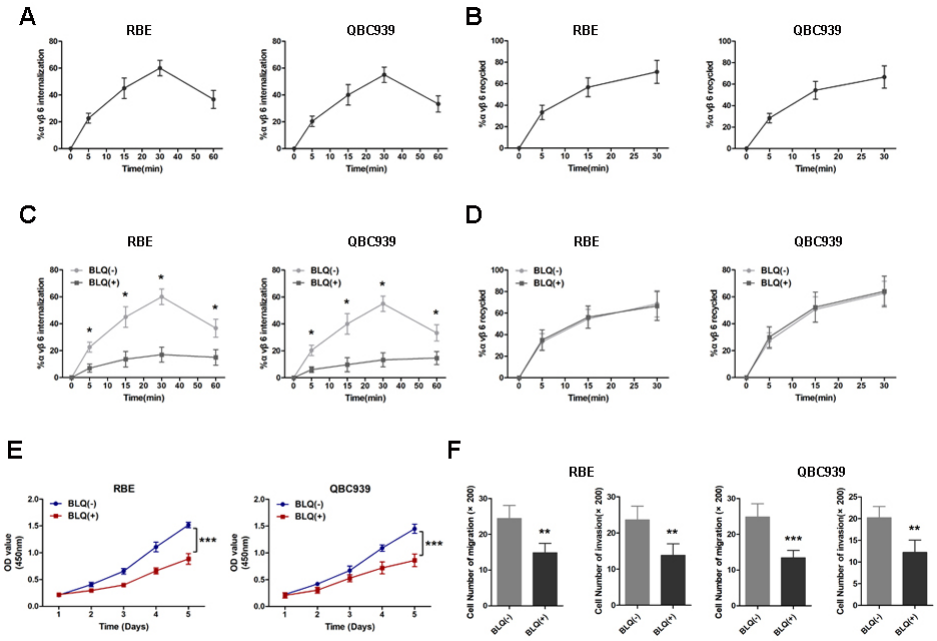

Figure 4. The effect of integrin αvβ6 trafficking in the progression of cholangiocarcinoma.

A. Internalization assays were conducted in RBE and QBC939 cells.

B. Recycling assays were conducted in RBE and QBC939 cells.

C. After the treatment with BLQ, internalization assays were conducted in cholangiocarcinoma cells.

D. Recycling assays were conducted in cholangiocarcinoma cells after BLQ treatment.

E. CCK-8 assays were conducted following BLQ treatment in cholangiocarcinoma cells.

F. Trans-well migration and invasion assays were conducted after BLQ treatment in cholangiocarcinoma cells. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Figure 5

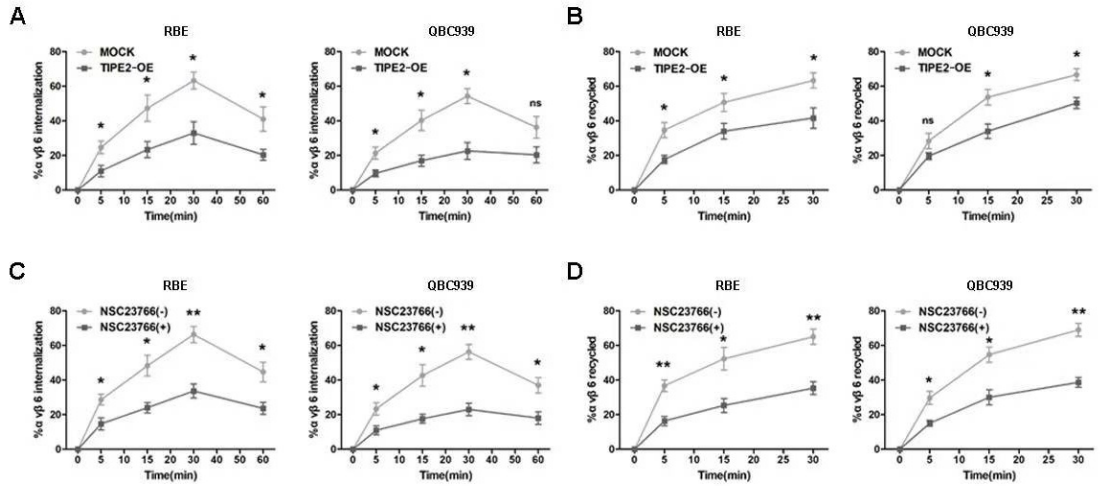

Figure 5. RAC1 was involved in the trafficking of integrin αvβ6.

A. Internalization assays were conducted in RBE and QBC939 cells after TIPE2 overexpression.

B. Recycling assays were conducted in RBE and QBC939 cells following TIPE2 overexpression.

C. Internalization assays were conducted in RBE and QBC939 cells that has been pre-treated with NSC23766.

D. Recycling assays were conducted in RBE and QBC939 cells that has been pre-treated with NSC23766. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Figure 6

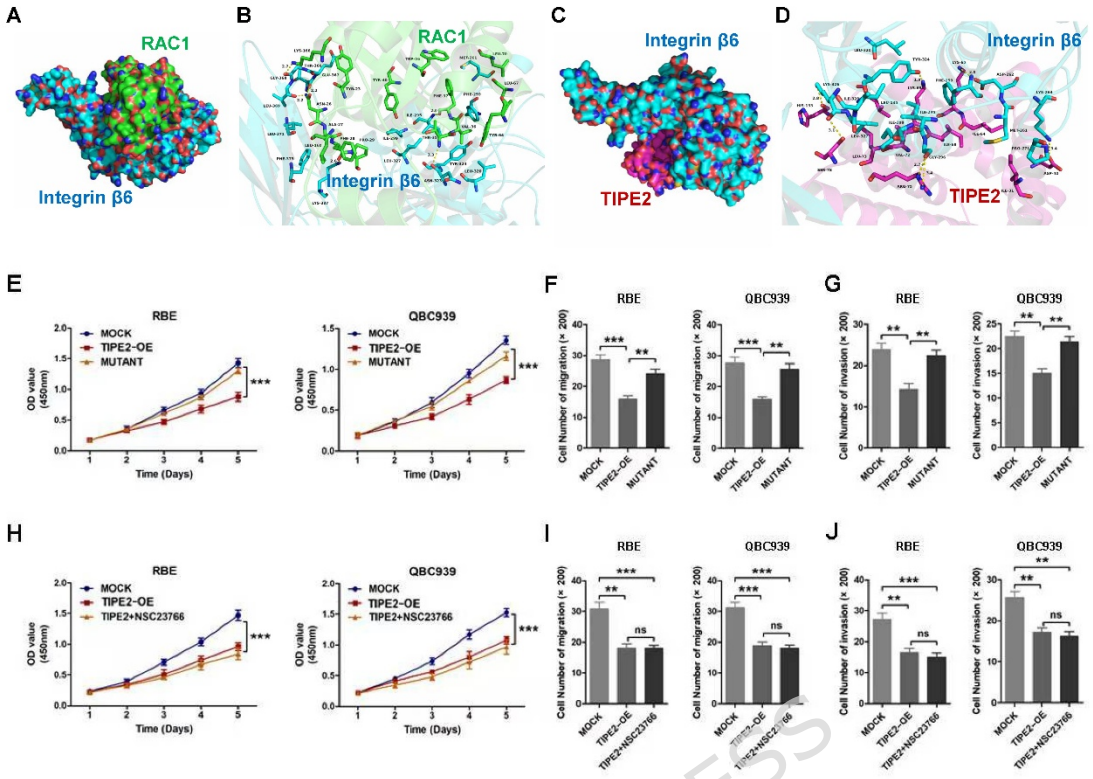

Figure 6. TIPE2 targeted RAC1 and suppressed the trafficking of integrin αvβ6.

A. Total view of the interaction between the integrin β6 (cyan) and RAC1 (green).

B. Detailed view of the interaction between the integrin β6 (cyan) and RAC1 (green).

C. Total view of the interaction between the TIPE2 (rose red) and the integrin β6 (cyan).

D. Detailed view of the interaction between the TIPE2 (rose red) and the integrin β6 (cyan).

E. CCK-8 assays were carried out in cholangiocarcinoma cells transfected with either the TIPE2 overexpression plasmid or the Mutant plasmid.

F. Trans-well migration assays were carried out in cholangiocarcinoma cells transfected with either the TIPE2 overexpression plasmid or the Mutant plasmid.

G. Trans-well invasion assays were carried out in cholangiocarcinoma cells transfected with either a TIPE2 overexpression plasmid or a Mutant plasmid.

H. CCK-8 assays were carried out after TIPE2 overexpression in cholangiocarcinoma cells with or without NSC23766 pre-treatment.

I. Trans-well migration assays were carried out after TIPE2 overexpression in cholangiocarcinoma cells with or without NSC23766 pre-treatment.

J. Trans-well invasion assays were carried out after TIPE2 overexpression in cholangiocarcinoma cells with or without NSC23766 pre-treatment. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Tables

Table 1. Correlation between TIPE2 expression and clinical characteristics of patients with cholangiocarcinoma.

Characteristic	Number	TIPE2 expression		χ2 value	*P*-value
		Low (%)	Hight (%)		
Age (years)				2.164	0.141
<65	136	69 (50.7)	67 (49.3)		
≥65	82	50 (61.0)	32 (39.0)		
Gender				0.027	0.870
Male	122	66 (54.1)	56 (45.9)		
Female	96	53 (55.2)	43 (44.8)		
Tumor location				2.151	0.341
iCCA	66	37 (56.1)	29 (43.9)		
hCCA	138	77 (55.8)	61 (44.2)		
eCCA	14	5 (35.7)	9 (64.3)		
Tumor size (cm)				10.913	**0.001***
≤3	112	49 (43.8)	63 (56.3)		
>3	106	70 (66.0)	36 (34.0)		
T stage				10.459	**0.015***
T1	16	5 (31.3)	11 (68.8)		
T2	152	78 (51.3)	74 (48.7)		
T3	37	26 (70.3)	11 (29.7)		
T4	13	10 (76.9)	3 (23.1)		
N stage				14.304	**<0.001***
N0	150	69 (46.0)	81(54.0)		
N1	68	50 (73.5)	18 (26.5)		
Distant metastasis				0.103	0.748

Yes	4	3 (75.0)	1 (25.0)		
No	214	116 (54.2)	98 (45.8)		
TNM stage				40.202	**<0.001***
I	20	3 (15.0)	17 (85.0)		
Ⅱ	107	46 (43.0)	61(57.0)		
Ⅲ	55	38 (69.1)	17 (30.9)		
Ⅳ	36	32 (88.9)	4 (11.1)		
Tumor differentiation				0.095	0.954
well	30	16 (53.3)	14 (46.7)		
moderately	152	84 (55.3)	68 (44.7)		
poorly	36	19 (52.8)	17 (47.2)		
Vascular invasion				20.686	**<0.001***
Yes	67	52 (77.6)	15 (22.4)		
No	151	67 (44.4)	84 (55.6)		

*eCCA: extrahepatic cholangiocarcinoma; hCCA: hilar cholangiocarcinoma; iCCA: intrahepatic cholangiocarcinoma