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Integrin $\beta 6$ serves as an immunohistochemical marker for lymph node metastasis and promotes cell invasiveness in cholangiocarcinoma

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Cholangiocarcinoma is a devastating malignancy that is notoriously difficult to diagnose and is associated with a high mortality. Despite extensive efforts to improve the diagnosis and treatment of this neoplasm, limited progress has been made. Integrin $\beta 6$ is a subtype of integrin that is expressed exclusively on the surfaces of epithelial cells and is associated with a variety of tumors. In the present study, we investigated the expression and roles of integrin $\beta 6$ in cholangiocarcinoma. $\beta 6$ upregulation in cholangiocarcinoma was correlated with lymph node metastasis and distant metastasis. Moreover, integrin $\beta 6$ was identified as a biomarker for the diagnosis of cholangiocarcinoma and an indicator of lymph node metastasis. Integrin $\beta 6$ significantly promoted the proliferation, migration and invasion of cholangiocarcinoma cells. Furthermore, integrin $\beta 6$ increased Rac1-GTPase, resulting in the upregulation of metalloproteinase-9 (MMP9) and F-actin polymerization. Taken together, our results indicate that integrin $\beta 6$ promotes tumor invasiveness in a Rac1-dependent manner and is a potential biomarker for tumor metastasis. Integrin $\beta 6$ may help to improve the diagnostic accuracy, and targeting $\beta 6$ may be a novel strategy for the treatment of cholangiocarcinoma.

Cholangiocarcinoma is neoplasm originating from the ductular epithelium of the biliary tree, either within the liver (intrahepatic cholangiocarcinoma) or more commonly from the extrahepatic bile ducts (extrahepatic cholangiocarcinoma). It was first reported by Durand Fardel in 1840, and the incidence of this rare disease is increasing^{1–5}. The major risk factors for cholangiocarcinoma are age, primary sclerosing cholangitis (PSC), chronic choledocholithiasis, hepatolithiasis, bile duct adenoma, and parasitic biliary infestation⁶. This disease is notoriously difficult to diagnose, and surgical resection is the current therapy of choice. The prognosis of cholangiocarcinoma is poor due to its late clinical presentation and the lack of effective non-surgical therapeutic modalities, with a 5-year overall survival rate of less than 5%, which has not significantly changed in the past 30 years^{7,8}. Therefore, a better understanding of the molecular mechanisms that are associated with the development of cholangiocarcinoma is needed for the treatment of this devastating disease.

Integrins are major cell adhesion receptors that mediate cell migration via cell–cell and cell–matrix interactions⁹. They belong to a family of a cell surface adhesion molecules and are composed of α and β subunits that are non-covalently associated¹⁰. Integrin $\alpha v \beta 6$ is the only heterodimer that the $\beta 6$ subunit can form, and it is expressed exclusively in epithelial cells. It is absent or weakly expressed in healthy adult epithelia, but it is highly expressed during embryogenesis, tissue repair and carcinogenesis^{11,12}. We have reported that $\beta 6$ integrin played an important role in the invasiveness, metastasis and degradation of the extracellular matrix of colorectal cancer, thyroid carcinoma, gastric carcinoma, and pancreatic carcinoma, and it has been identified as an independent unfavorable prognostic indicator in a number of human cancers^{11,13–19}. In addition, our previous research

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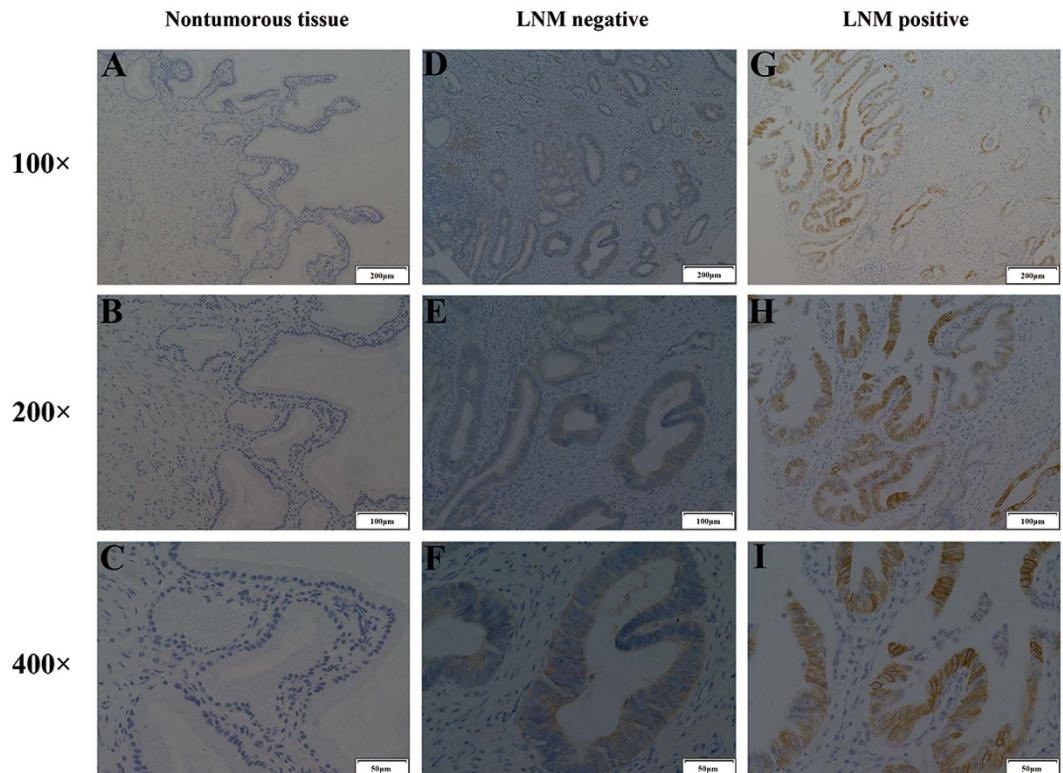


Figure 1. The expression of integrin $\beta 6$ in non-tumorous bile duct tissues and cholangiocarcinoma tissues. (A–C) Negative $\beta 6$ expression in non-tumorous bile duct tissues. (D–F) Moderate expression of integrin $\beta 6$ in cholangiocarcinoma tissues without lymph node metastasis. (G–I) Strong staining of $\beta 6$ in cholangiocarcinoma tissues with lymph node metastasis.

demonstrated that there is a direct link between extracellular signal-regulated kinase (ERK2) and $\beta 6$ integrin, which is essential for $\beta 6$ -mediated ERK2 activation and the corresponding downstream effects^{20,21}. Furthermore, we synthesized $\beta 6$ -targeted immunoliposomes and demonstrated that they had favorable antitumor efficacy and insignificant systemic toxicity in colon cancer²². Unsurprisingly, the exclusive expression of integrin $\beta 6$ in epithelium-derived tumors and its important effects make it a novel therapeutic target for the treatment of many tumors. As cholangiocarcinoma originates from the ductular epithelium of the biliary tree, the expression and roles of integrin $\beta 6$ in cholangiocarcinoma should be further explored.

Rac1 is a member of the Rho family of small GTPases. It is highly expressed in many cancer cell lines and has been implicated in a wide variety of cellular processes, including cytoskeletal reorganization and gene transcription, and its overexpression is correlated with poor prognosis in cholangiocarcinoma^{23,24}. Furthermore, Rac1 also regulates various downstream effector molecules related to tumor invasiveness, such as MMP9 and uPA, and it is considered a central regulator of tumor malignancy^{25,26}.

In the present study, for the first time, we demonstrated that integrin $\beta 6$ is overexpressed in cholangiocarcinoma and is associated with lymph node metastasis. Our data also indicate that integrin $\beta 6$ is crucial in the malignant progress of cholangiocarcinoma. Moreover, integrin $\beta 6$ promotes the development of cholangiocarcinoma by activating Rac1. Our data indicated that integrin $\beta 6$ may contribute to improving the diagnostic accuracy and treatment of cholangiocarcinoma and should be further explored.

Results

Integrin $\beta 6$ expression was associated with lymph node metastasis and distant metastasis in cholangiocarcinoma.

To investigate the expression of integrin $\beta 6$ in cholangiocarcinoma, we performed an immunohistochemistry (IHC) analysis using a total of 95 paraffin-embedded cholangiocarcinoma samples and corresponding adjacent normal tissues. As shown in Fig. 1, integrin $\beta 6$ staining was negative in non-tumorous tissues (Fig. 1A–C), while moderate expression was observed in tumor tissues (Fig. 1D–F). Furthermore, strong $\beta 6$ expression was observed in specimens with lymph node metastasis (Fig. 1G–I). Integrin $\beta 6$ was predominantly expressed in the membrane of the tumor cells. To further explore the clinical significance of $\beta 6$ expression in cholangiocarcinoma, we analyzed the correlation between $\beta 6$ expression and various clinicopathological factors. As shown in Table 1, no significant association was observed between $\beta 6$ expression and age ($P = 0.5226$), gender ($P = 0.6637$), CA19-9 level ($P = 0.2648$), CEA level ($P = 0.6670$), depth of invasion ($P = 0.4396$) and tumor differentiation ($P = 0.3684$). However, $\beta 6$ expression was significantly associated with lymph node metastasis ($P = 0.0003$) and distant metastasis ($P = 0.0126$), indicating that $\beta 6$ may be involved in the progression of cholangiocarcinoma.

Variable	Number	$\beta 6$ expression		P value
		Low	High	
Age (years)				
<60	42	13	29	0.5226
≥ 60	53	20	33	
Gender				
Male	57	21	36	0.6637
Female	38	12	26	
CA19-9 level				
<37 ku/ml	35	15	20	0.2648
≥ 37 ku/ml	60	18	42	
CEA level				
<15 ng/ml	40	15	25	0.6670
>15 ng/ml	55	18	37	0.4396
Depth of invasion (T)				
Tis	3	2	1	
T1	16	7	9	
T2	38	13	25	
T3	36	10	26	
Missing	2	1	1	
Lymph node metastasis (LNM)				
Negative	34	20	14	0.0003
Positive	61	13	48	
Distant metastasis (M)				
Negative (M0)	72	30	42	0.0126
Positive (M1)	23	3	20	
Tumor differentiation				
Well	15	3	12	0.3684
Moderate	37	12	25	
Poor	40	16	24	
Missing	3	2	1	

Table 1. Correlations between $\beta 6$ expression and clinicopathological characteristics in cholangiocarcinoma.

Integrin $\beta 6$ was a promising biomarker for diagnosis of cholangiocarcinoma and prediction of lymph node metastasis. As shown in Fig. 2A, $\beta 6$ staining was negative in almost all non-tumorous tissues, while moderate to strong staining was observed in cholangiocarcinoma tissues, suggesting that $\beta 6$ can be used for the diagnosis of cholangiocarcinoma. The IHC results showed that $\beta 6$ expression was significantly upregulated in tumor tissues with lymph node metastasis compared to that of samples without lymph node metastasis (Fig. 2B).

To determine the diagnostic value of $\beta 6$ expression in cholangiocarcinoma, we constructed receiver operator characteristic (ROC) curves. To assess the capacity of $\beta 6$ expression (IHC sum scores) to diagnose cholangiocarcinoma or to predict lymph node metastasis, we calculated the area under the curve (AUC). The ROC curves suggested that the AUC value for the diagnosis of cholangiocarcinoma was up to 0.876 (Fig. 2C, CI (95%): 0.822 to 0.929, $P < 0.001$), with an estimated sensitivity and specificity of 78.95% and 93.68%, respectively, for $\beta 6$ expression as a predictor of cholangiocarcinoma (Supplementary Table S1). Moreover, the AUC value for predicting lymph node metastasis in cholangiocarcinoma was 0.701 (Fig. 2D, CI (95%): 0.599 to 0.791, $P < 0.001$), with an estimated sensitivity and specificity of 77.55% and 63.04%, respectively (Supplementary Table S2).

Integrin $\beta 6$ markedly promoted the proliferation, migration and invasion of cholangiocarcinoma cells. As integrin $\beta 6$ was highly expressed in cholangiocarcinoma, the functional roles of integrin $\beta 6$ in human cholangiocarcinoma cells were investigated. $\beta 6$ -specific siRNA was used in RBE and QBC939 cells. First, the efficiency of $\beta 6$ silencing in these cells was determined. As shown in Fig. 3A,B, $\beta 6$ expression was significantly decreased at both the mRNA level and protein level after transfection with $\beta 6$ -specific siRNA. CCK8 assays were used to evaluate the effects of integrin $\beta 6$ on the viability of cholangiocarcinoma cells. The results showed that $\beta 6$ silencing in RBE and QBC939 cells markedly suppressed cell viability 48 hours after transfection in a time-dependent manner (Fig. 3C). Then, MTT assays were conducted, and the results indicated that $\beta 6$ downregulation in RBE and QBC939 cells was associated with decreased cell proliferation (Fig. 3D). Wound-healing assays showed that the migration of RBE and QBC939 cells was markedly suppressed after downregulation of $\beta 6$ (Fig. 3E). Then, we performed Transwell migration and invasion assays in cholangiocarcinoma cells after $\beta 6$ silencing. $\beta 6$ silencing had no obvious effect on cell viability within 12 hours, which eliminated the potential confounding influence of $\beta 6$ silencing-induced cell proliferation on cell migration and invasion. The results showed

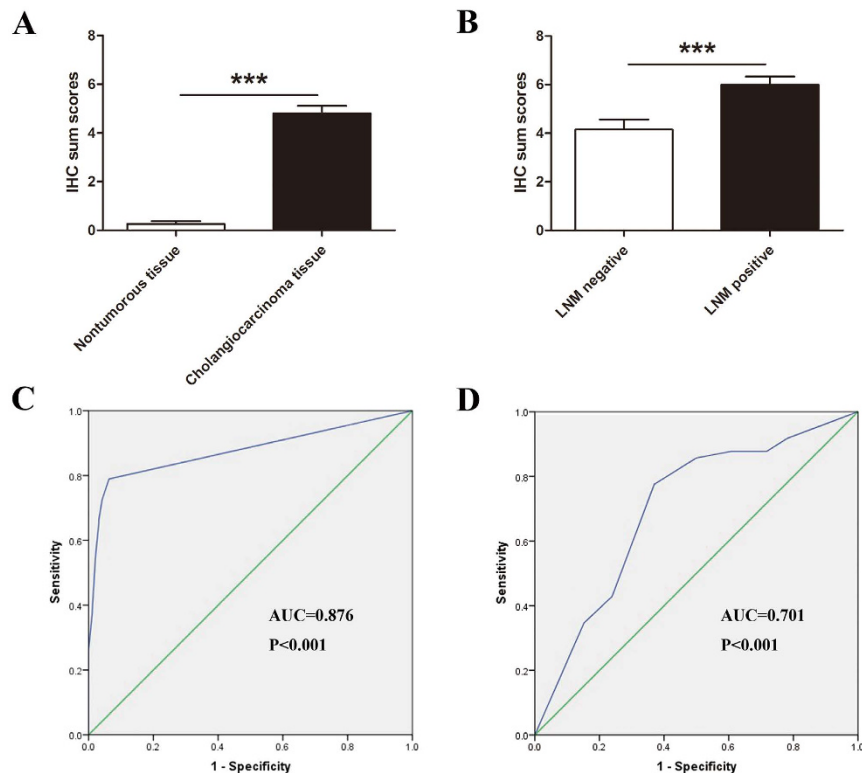


Figure 2. Upregulation of $\beta 6$ in more aggressive cholangiocarcinoma tissues and ROC curves to assess the diagnostic value of $\beta 6$ expression in cholangiocarcinoma. IHC sum scores were used to assess integrin $\beta 6$ expression in cholangiocarcinoma tissues and adjacent non-tumorous tissues. $\beta 6$ expression was significantly upregulated in cholangiocarcinoma tissues compared to adjacent non-tumorous tissues. (A) $\beta 6$ expression was markedly higher in cholangiocarcinoma tissues with lymph node metastasis than those without lymph node metastasis. (B) The ROC curves showed strong separation between cholangiocarcinoma tissues and adjacent non-tumorous tissues, with an AUC of 0.989 ($P < 0.001$). (C) The ROC curves showed strong separation between the patients with and without lymph node metastasis, with an AUC of 0.741 ($P < 0.001$). *** $P < 0.001$.

that the migration and invasion capacities of both RBE and QBC939 cells were significantly suppressed by $\beta 6$ silencing (Fig. 3E,G).

To further confirm the effects of $\beta 6$ on cell proliferation, migration and invasion in cholangiocarcinoma cells, we repeated the aforementioned experiments after upregulation of $\beta 6$ using a $\beta 6$ overexpression plasmid. As shown in Fig. 4A,B, $\beta 6$ expression was significantly increased after transfection. The results showed that $\beta 6$ overexpression markedly promoted the proliferation, migration and invasion of cholangiocarcinoma cells (Fig. 4C–G).

These data suggested that integrin $\beta 6$ could promote the proliferation, migration and invasion of cholangiocarcinoma cells.

Integrin $\beta 6$ induced the expression of MMP9 in cholangiocarcinoma cells. To identify the mechanism underlying the effects of $\beta 6$ on invasion in cholangiocarcinoma cells, we measured the mRNA expression of uPA, MMP2, MMP3, and MMP9 using quantitative real-time PCR after silencing or overexpressing $\beta 6$. We found that $\beta 6$ -specific siRNA significantly downregulated MMP9 mRNA expression in cholangiocarcinoma cells, and $\beta 6$ overexpression markedly increased MMP9 mRNA expression. No effect was observed on uPA, MMP2 and MMP3 (Fig. 5A–D).

We further explored the effect of $\beta 6$ on secreted MMP9 in the supernatant of cholangiocarcinoma cells by ELISA. Consistent with the results from the mRNA analysis, the data showed that the secreted MMP9 in the supernatant of cholangiocarcinoma cells was significantly decreased after suppression of $\beta 6$ expression (Fig. 5E), while $\beta 6$ overexpression increased MMP9 secretion in the supernatant (Fig. 5F). Taken together, these results revealed that integrin $\beta 6$ could induce MMP9 expression at both the mRNA and protein levels.

Integrin $\beta 6$ activated Rac1 in cholangiocarcinoma cells. As previous studies showed that Rac1 activity is involved in the malignant behaviors of cancer cells²⁴, we hypothesized that integrin $\beta 6$ may promote invasiveness by activating Rac1. To confirm our hypothesis, we performed four experiments. First, using the specific Rac1 activity inhibitor NSC23766, we found that Rac1 was essential for the migration and invasion of cholangiocarcinoma cells, as NSC23766 suppressed migration and invasion of RBE cells in a dose-dependent manner (Fig. 6A). Then, we demonstrated that overexpression of integrin $\beta 6$ could effectively promote the migration and

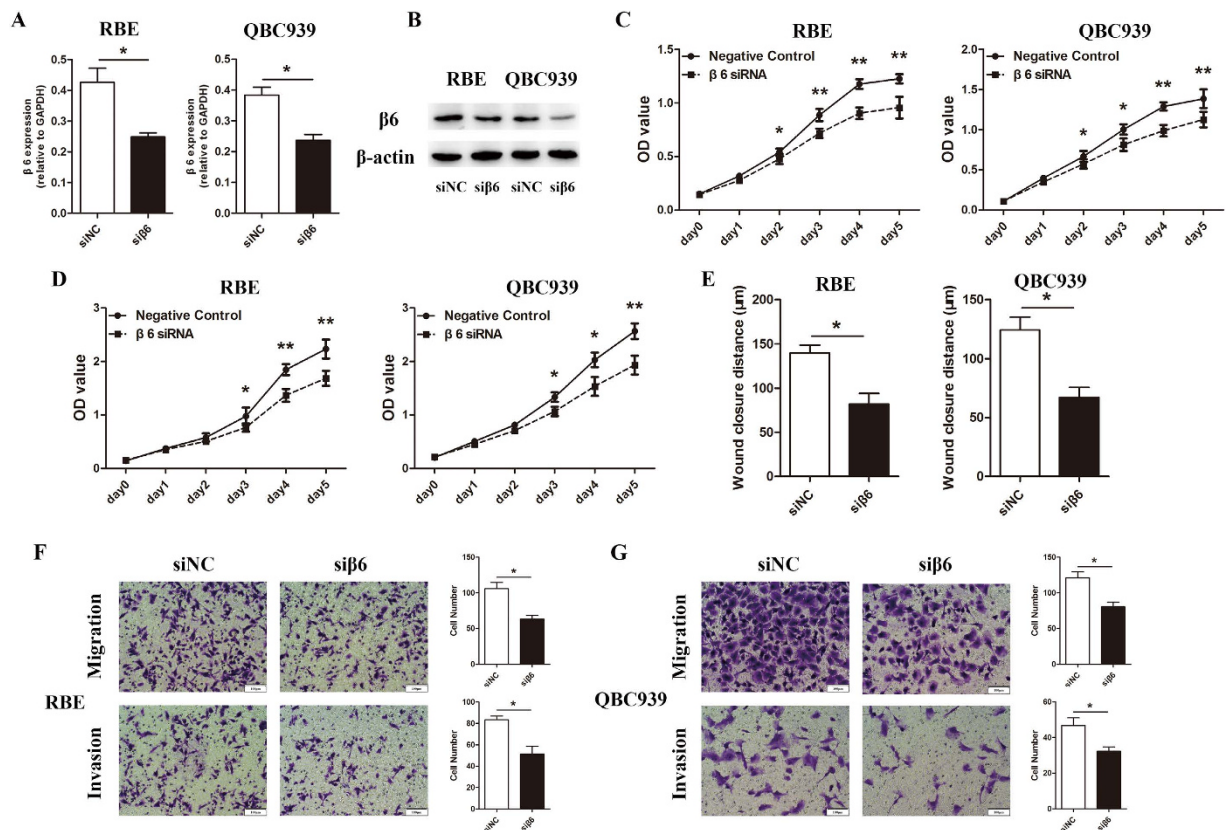


Figure 3. $\beta 6$ silencing markedly decreased the proliferation, migration and invasion of cholangiocarcinoma cells. (A) Real-time PCR showed that integrin $\beta 6$ expression was high in both RBE and QBC939 cells; $\beta 6$ expression was significantly suppressed after $\beta 6$ -specific siRNA transfection. (B) Western blotting showed that $\beta 6$ expression was significantly suppressed after $\beta 6$ siRNA transfection in RBE and QBC939 cells at the protein level. (C) CCK8 assays revealed that silencing of $\beta 6$ significantly decreased cell viability in RBE and QBC939 cells. (D) After transfection of $\beta 6$ siRNA, MTT assays were conducted to evaluate the proliferation of RBE and QBC939 cells. (E) RBE and QBC939 cells transfected with $\beta 6$ siRNA were used for wound-healing assays. (F,G) RBE (F) and QBC939 (G) cells transfected with $\beta 6$ siRNA were used for Transwell migration assays and invasion assays. Five fields of cells in the lower chamber were counted ($\times 200$ magnification). Data represent the mean \pm SD of three independent experiments. * $P < 0.05$; ** $P < 0.01$.

invasion capacity of cholangiocarcinoma cells, whereas NSC23766 significantly inhibited this effect (Fig. 6B). Moreover, the $\beta 6$ -mediated promotion of migration and invasion could be inhibited by specific Rac1 siRNA (Fig. 6C). In addition, we found that $\beta 6$ silencing significantly decreased Rac1-GTPase activity (Fig. 6D), while $\beta 6$ overexpression increased Rac1 activity in cholangiocarcinoma cells (Fig. 6E). All these results demonstrated that $\beta 6$ promoted migration and invasion by activating Rac1.

The effects of integrin $\beta 6$ were mediated through Rac1 downstream effectors, F-actin polymerization and MMP9 expression in cholangiocarcinoma cells. Previous studies found that Rac1 activity is important for microfilament dynamics, which directly regulate cell motility²⁷. In the present study, we explored the effect of integrin $\beta 6$ on the microfilament dynamics in cholangiocarcinoma cells. Figure 7A shows that in the siNC group, F-actin was organized into the cytoskeleton, while in the $\beta 6$ -silenced group, F-actin was depolymerized in most cells. The results revealed that F-actin was significantly depolymerized after treatment with $\beta 6$ siRNA. Moreover, $\beta 6$ overexpression increased F-actin polymerization, and after treatment with NSC23766, this effect was abolished (Fig. 7B), indicating that $\beta 6$ promoted F-actin polymerization by activating Rac1.

Our previous work showed that $\beta 6$ induced the expression of MMP9 and promoted the invasiveness of cholangiocarcinoma cells in a Rac1-dependent manner. We thus hypothesized that integrin $\beta 6$ induced the expression of MMP9 via the Rac1 pathway. To test our hypothesis, we explored the effect of Rac1 on $\beta 6$ -induced MMP9 expression. Rac1 silencing significantly decreased the expression of MMP9 at both the mRNA and protein level (Fig. 7C,D). Moreover, $\beta 6$ silencing decreased MMP9 expression, while $\beta 6$ overexpression increased the expression of MMP9. However, after pretreatment with a Rac1 activity inhibitor, the effect of both $\beta 6$ silencing and overexpression was abolished (Fig. 7E). These results confirmed our hypothesis and indicated that integrin $\beta 6$ induced the expression of MMP9 by activating Rac1.

Integrin $\beta 6$ promoted tumor growth and MMP9 expression of tumor cells in nude mice. To evaluate the effect of integrin $\beta 6$ *in vivo*, we established a subcutaneous xenograft tumor model in nude mice

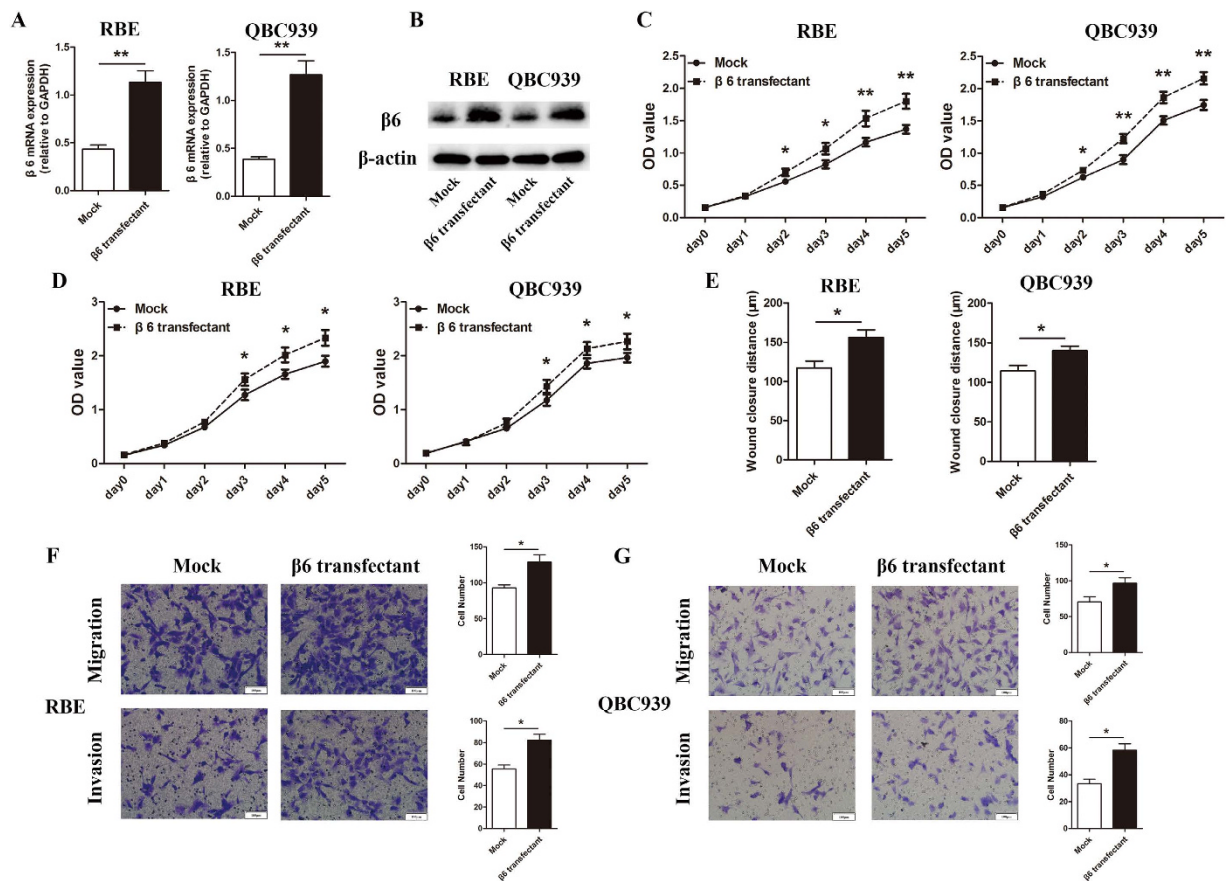


Figure 4. $\beta 6$ overexpression markedly promoted the proliferation, migration and invasion of cholangiocarcinoma cells. (A) Real-time PCR showed that integrin $\beta 6$ expression was significantly upregulated after transfection of the $\beta 6$ overexpression plasmid. (B) Western blotting showed that $\beta 6$ expression was significantly increased after transfection with the $\beta 6$ overexpression plasmid in RBE and QBC939 cells at the protein level. (C) CCK8 assays revealed that overexpression of $\beta 6$ significantly promoted cell viability in RBE and QBC939 cells. (D) After $\beta 6$ overexpression, MTT assays were conducted to evaluate the proliferation of RBE and QBC939 cells. (E) RBE and QBC939 cells transfected with the $\beta 6$ overexpression plasmid were used for wound-healing assays. (F,G) RBE (F) and QBC939 (G) cells transfected with the $\beta 6$ overexpression plasmid were used for Transwell migration assays and invasion assays. Five fields of cells were counted in the lower chambers ($\times 200$ magnification). Data represent the mean \pm SD of three independent experiments. * $P < 0.05$; ** $P < 0.01$.

bearing RBE cells. RBE cells were infected with the lentiviral vectors LV-negative control, LV-si $\beta 6$ or LV- $\beta 6$. Consistent with our *in vitro* findings, our results showed that silencing of $\beta 6$ markedly suppressed the growth of RBE xenograft tumors compared with that of the NC group, while $\beta 6$ overexpression promoted the growth of tumors (Fig. 8A). The overall mean tumor volume and tumor weight of the LV-si $\beta 6$ group were significantly smaller than those of the LV-NC group, whereas tumors in the LV- $\beta 6$ group had a relatively larger volume and weight (Fig. 8B,C). As shown in Fig. 8D,E, compared with the LV-NC group, $\beta 6$ silencing markedly decreased the MMP9 expression in the xenograft tumors, while $\beta 6$ overexpression significantly increased the MMP9 expression. These results indicated that integrin $\beta 6$ promoted the growth and MMP9 expression of cholangiocarcinoma xenograft tumors.

Discussion

Cholangiocarcinoma is a predominantly fatal cancer, which is usually difficult to diagnose and to treat⁷. Therefore, elucidating the mechanisms underlying the malignant behavior of cholangiocarcinoma may facilitate the development of clinical strategies. In the present study, $\beta 6$ expression was markedly upregulated in cholangiocarcinoma and was associated with lymph node metastasis. Integrin $\beta 6$ promoted the migratory and invasive capacities of cholangiocarcinoma cells by activating Rac1, indicating that integrin $\beta 6$ may be a potential target for the treatment of cholangiocarcinoma.

The epithelial-restricted integrin $\beta 6$ is generally upregulated in tissues that have undergone malignant transformation^{28,29}. Increased expressions of $\beta 6$ were previously reported in carcinomas of the colon, breast, stomach and pancreas, and increased $\beta 6$ expression is associated with advanced tumor stage and poor prognosis^{13,17,18,30}. Patsenker *et al.* reported that integrin $\beta 6$ is a highly specific immunohistochemical marker for the differential diagnosis of hepatocellular carcinoma (HCC) and cholangiocarcinoma, as $\beta 6$ is strongly expressed in

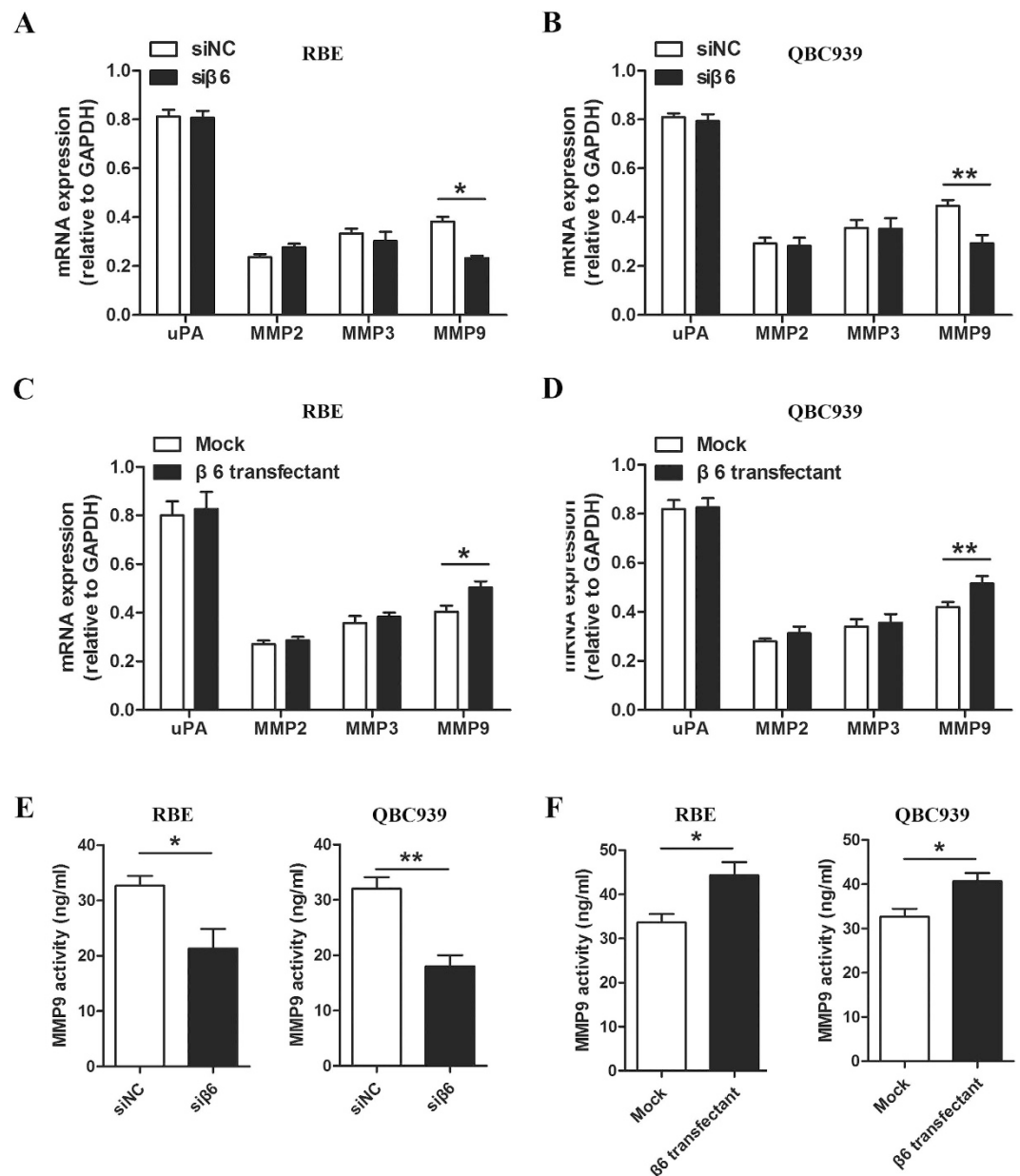


Figure 5. Integrin $\beta 6$ increased MMP9 expression at both the mRNA and protein levels. (A,B) MMP9 mRNA was significantly decreased in RBE cells (A) and QBC939 cells (B) after transfection with $\beta 6$ siRNA, while the expression of uPA, MMP2 and MMP3 showed no significant changes. (C,D) MMP9 mRNA was significantly increased in RBE cells (C) and QBC939 cells (D) after $\beta 6$ overexpression, while the expression of uPA, MMP2 and MMP3 showed no significant changes. (E) The protein level of MMP9 was detected by ELISA in RBE and QBC939 cells transfected with $\beta 6$ siRNA. (F) The protein level of MMP9 was detected by ELISA in RBE and QBC939 cells after $\beta 6$ overexpression. Data represent the mean \pm SD from three independent experiments. * $P < 0.05$; ** $P < 0.01$.

cholangiocarcinoma, and in HCC, $\beta 6$ staining is negative³¹. Here, we reported that the increased $\beta 6$ expression was related to lymph node metastasis and distant metastasis, suggesting that in addition to an immunohistochemical marker, $\beta 6$ staining in the surgical specimens could be used to evaluate the risks of lymph node metastasis. Additionally, integrin $\beta 6$ may also be involved in the development of cholangiocarcinoma.

Integrin $\beta 6$ promotes malignant behaviors, such as cell proliferation, migration, invasion and metastasis, in many types of malignancies^{15,17,21,30}. However, the role of integrin $\beta 6$ in cholangiocarcinoma cells has not been elucidated. Our results demonstrated that $\beta 6$ could significantly promote cell proliferation and invasion.

Previous studies showed that $\beta 6$ increases the expression and secretion of MMP2, MMP3 and MMP9, resulting in the degradation of the extracellular matrix and increased tumor cell invasiveness in colon cancer, pancreatic cancer and ovarian cancer^{32,33}. MMP9 is highly expressed in tissues, and it was shown to be a valuable prognostic indicator in cholangiocarcinoma³⁴. As $\beta 6$ also promoted the invasiveness of cholangiocarcinoma cells,

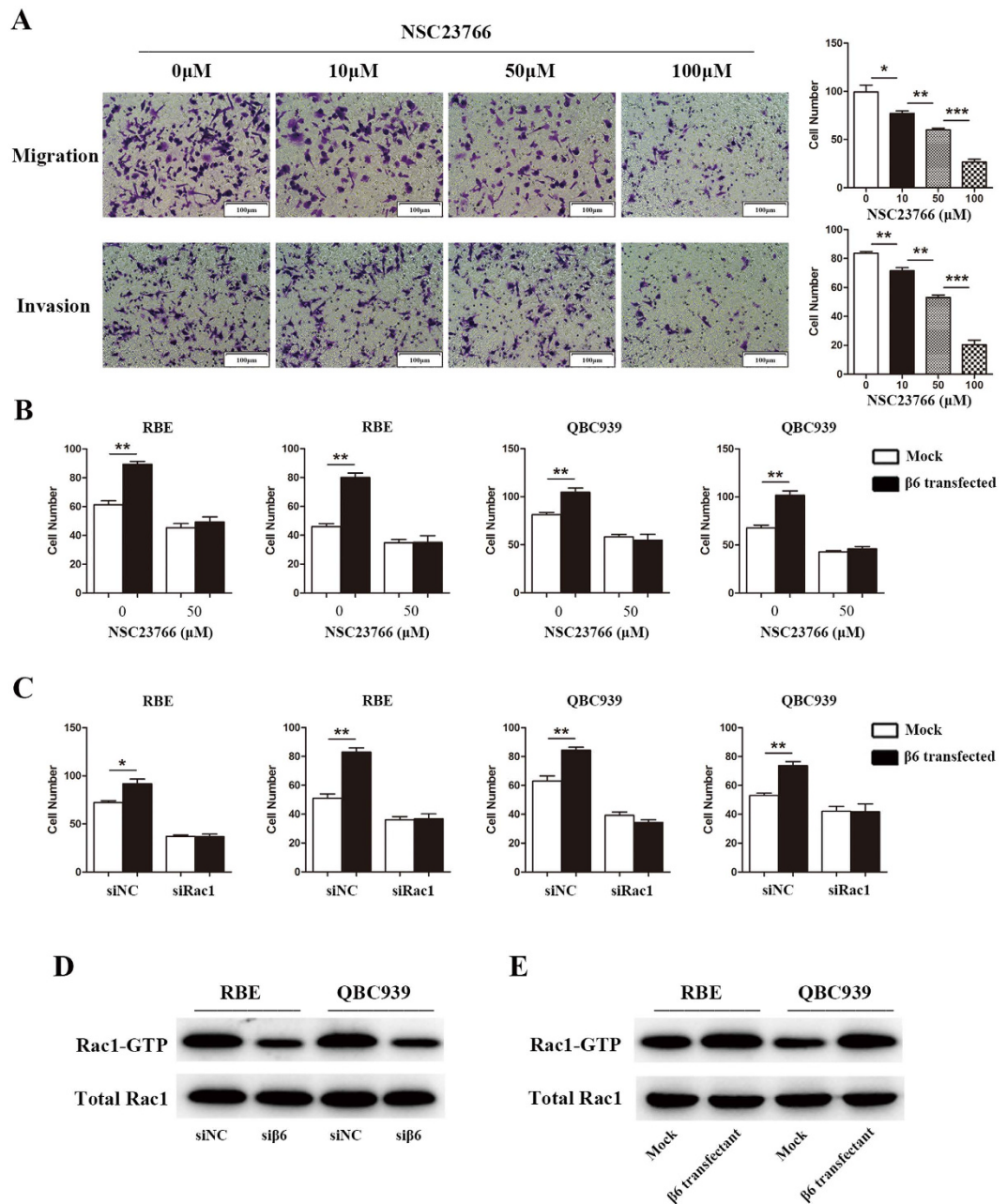


Figure 6. Integrin β 6 promoted the invasiveness of cholangiocarcinoma cells by activating Rac1. (A) RBE cells treated with different concentrations of NSC23766, a Rac1 inhibitor, were used for migration and invasion assays. (B) After treatment with NSC23766, RBE and QBC939 cells were transfected with the β 6-overexpressing plasmid, and the migration and invasion capacities were measured by Transwell assays. (C) RBE and QBC939 cells co-transfected with Rac1-specific siRNA and β 6-overexpressing plasmid were used for migration and invasion assays. (D) After transfection with β 6 siRNA or β 6-overexpressing plasmid, cell lysates of RBE and QBC939 were used for PAK-GST pull down assays. Activated Rac1 as well as total Rac1 pulled down in the lysates were detected by western blot analysis. Data are shown as the mean \pm SD, and the results shown are representative of 3 independent experiments. Five fields of cells in the lower compartment were counted in Transwell assays (200 \times magnification). * P < 0.05; ** P < 0.01; *** P < 0.001.

we explored the effect of β 6 on MMP expression in cholangiocarcinoma. Additionally, the effect of β 6 on uPA was assessed due to its role in cell invasiveness, and uPA was highly expressed in cholangiocarcinoma cells. Our results showed that β 6 induced the expression of MMP9 but not that of uPA, MMP2 or MMP3 in cholangiocarcinoma cells. The detailed mechanism by which β 6 promotes tumor development is complicated. Integrin β 6 may function through the protein kinase C (PKC) pathway in colon cancer, while β 6 may also bind ERK and subsequently activate the ERK-Ets1 pathway in colon cancer and pancreatic cancer³⁵. The SDF-1/CXCR4 axis and IL-8 are also involved in the β 6-regulated tumor cell migration^{15,36}. Rac1 plays a vital role in the development of

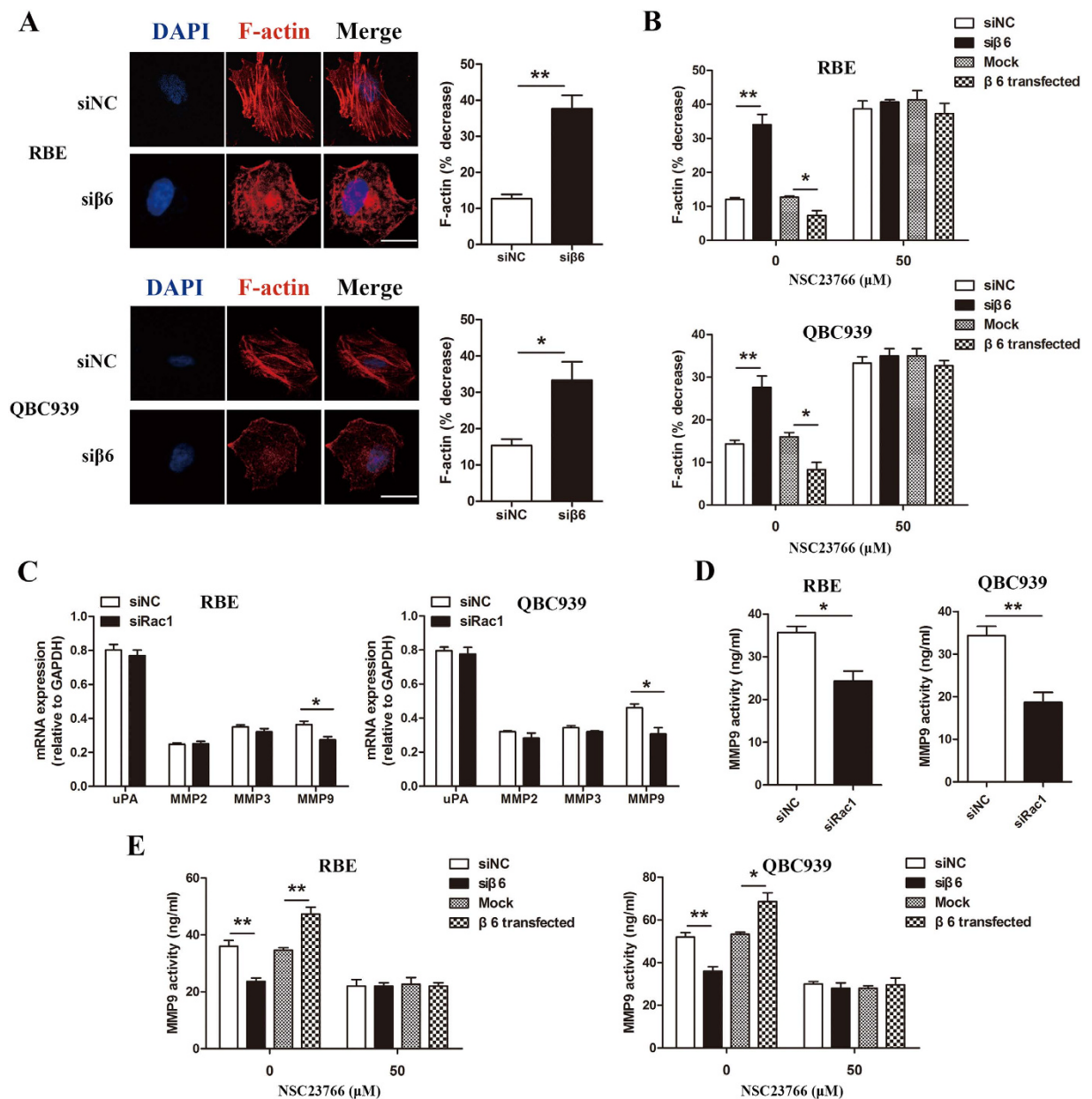


Figure 7. Integrin $\beta 6$ induced F-actin polymerization and MMP9 expression by activating Rac1. (A) After transfection of $\beta 6$ siRNA, F-actin in RBE and QBC939 cells was stained by TRITC-conjugated phalloidin and observed with laser confocal microscopy. The % decrease in F-actin = [(F-actin in untreated cells - F-actin in siNC or $\beta 6$ siRNA treated cells)/F-actin in untreated cells] \times 100. Scale bar, 10 μ m. (B) After treatment with NSC23766, RBE and QBC939 cells were transfected with $\beta 6$ siRNA or $\beta 6$ -overexpressing plasmid, and F-actin was stained by TRITC-conjugated phalloidin and observed with laser confocal microscopy. (C) MMP9 mRNA expression was significantly decreased in RBE cells and QBC939 cells after transfection with Rac1 siRNA, while the expression of uPA, MMP2 and MMP3 showed no significant changes. (D) The protein level of MMP9 was detected by ELISA in RBE and QBC939 cells after Rac1 silencing. (E) The protein level of MMP9 was detected by ELISA in RBE (C) and QBC939 cells (D) pretreated with NSC23766 and $\beta 6$ siRNA or $\beta 6$ -overexpressing plasmid. Data represent the mean \pm SD from three independent experiments. * P < 0.05; ** P < 0.01.

cholangiocarcinoma^{24,37}. Rac1 is a member of the Rho family of small GTPases and has been implicated in a wide variety of cellular processes, especially cell motility and cytoskeleton polymerization. Rac1 could also induce the expression and secretion of MMPs^{23,25,38,39}. Therefore, targeting Rac1 may be a promising therapeutic approach for the treatment of cholangiocarcinoma. MMP9 was reported to contribute to the cholangiocarcinogenesis in a Rac1-dependent manner⁴⁰. Consistent with these results, our data demonstrated that $\beta 6$ promoted the invasiveness of cholangiocarcinoma cells by activating Rac1, as both NSC23766 and Rac1 siRNA could abolish the effect of $\beta 6$. In addition, $\beta 6$ could activate Rac1, but it had no effect on total Rac1 expression. Mechanistically, we demonstrated that $\beta 6$ facilitated F-actin polymerization and induced MMP9 expression by activating Rac1. These

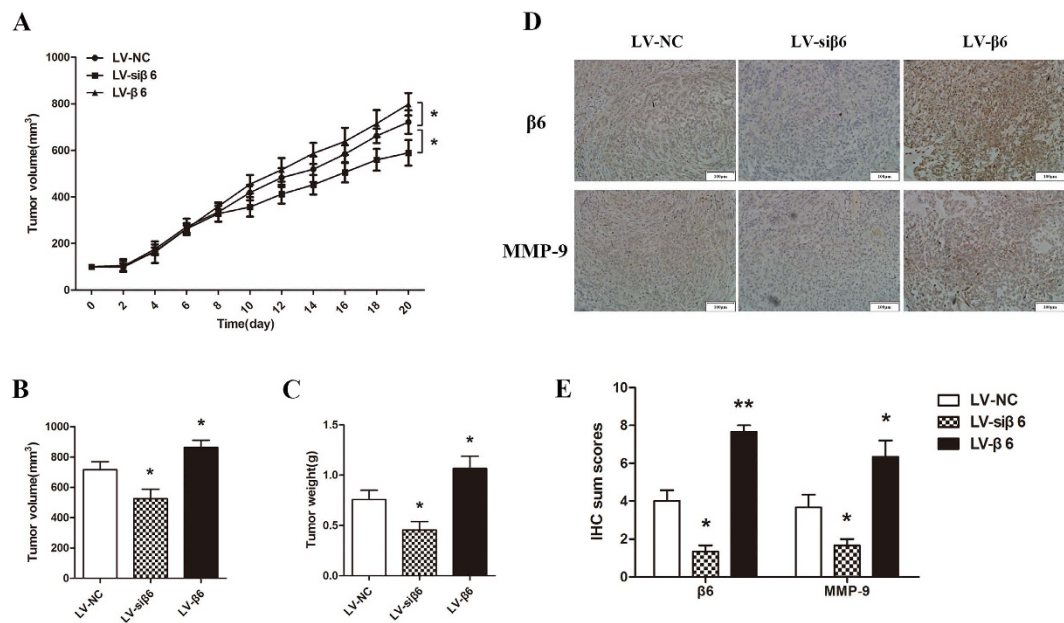


Figure 8. Integrin $\beta 6$ effectively promoted tumor growth and MMP9 expression of subcutaneous xenograft tumors. The growth curves of tumors from the LV-NC, LV-si $\beta 6$ and LV- $\beta 6$ groups. Tumors were isolated and measured. The mean volume of tumors in the LV-si $\beta 6$ group ($n = 6$) was significantly smaller than that of the control, while the LV- $\beta 6$ group ($n = 6$) had a larger mean tumor volume. (A) The mean tumor weight was significantly lower in the LV-si $\beta 6$ group ($n = 6$) and higher in the LV- $\beta 6$ group ($n = 6$) compared to the control. (B) The expression of integrin $\beta 6$ and MMP9 was detected by IHC in paraffin-embedded tumor tissue sections. (C) IHC sum scores were used to determine the expression of integrin $\beta 6$ and MMP9 in the xenograft tumor tissues. Data represent the mean \pm SD. * $P < 0.05$; ** $P < 0.01$.

results indicated that integrin $\beta 6$ is an endogenous activator of Rac1 in cholangiocarcinoma, which contributed to the malignant behaviors of cholangiocarcinoma cells.

Previous studies reported that integrin $\beta 6$ could localize to the cytoplasm and activate ERK via a specific binding site. Then, activated ERK translocates to the nucleus and activates Ets-1, leading to the proliferation, migration, and invasion of tumor cells^{20,32,35}. Here, we demonstrated that $\beta 6$ promoted tumor cell malignant behaviors by activating Rac1. Rac1 is important in regulating vesicular transport. Therefore, the effect of Rac1 on the internalization of $\beta 6$ needs to be further explored.

Cholangiocarcinoma is a severe disease with poor prognosis. Molecular targeting strategies to improve treatment and reduce the side effects of conventional chemotherapy and radiotherapy are needed. In the present study, we demonstrated that integrin $\beta 6$ upregulation was an unfavorable indicator in cholangiocarcinoma. By targeting and activating Rac1, $\beta 6$ promotes the development and progression of cholangiocarcinoma. The perturbation of integrin $\beta 6$ may present a potential strategy for the treatment of patients suffering from cholangiocarcinoma.

Materials and Methods

Tissue samples. A total of 95 human cholangiocarcinoma specimens and the corresponding adjacent normal specimens were collected from Qilu Hospital of Shandong University. Among the 95 cholangiocarcinoma specimens, 61 cases had lymph node metastasis, while 34 cases had no lymph node metastasis. Clinicopathological classification and staging were determined according to the American Joint Committee on Cancer (AJCC) criteria⁴¹. All samples were fixed in 40 g/L formaldehyde and were embedded in paraffin for histological diagnosis and immunohistochemical studies. The protocols in the study were carried out in accordance with the approved guidelines. All the experimental protocols were approved by the Ethics Committee of Shandong University, China. Written informed consent was obtained from all subjects.

Cell culture and transfection. The human cholangiocarcinoma cell lines, RBE and QBC939, obtained from the American Type Culture Collection (ATCC), were separately maintained in RPMI 1640 and DMEM medium, respectively, (Gibco, CA, USA) supplemented with 10% inactivated fetal bovine serum (FBS) (Gibco, CA, USA) in a humidified cell incubator with an atmosphere of 5% CO₂ at 37 °C. Both RBE and QBC939 cells were transfected with $\beta 6$ siRNA or a $\beta 6$ overexpression plasmid using Lipofectamine 2000 according to the manufacturer's protocols (Invitrogen, Carlsbad, CA, USA). The $\beta 6$ overexpression plasmid was constructed as previously described²⁰. The $\beta 6$ siRNA primers were purchased from GenePharma and were as follows: 5'-GCUAAAGGAUGUCAUUAATT-3', reverse 5'-UUAAUUGACAUCUUUAGCTA-3'. The Rac1-siRNA were as follows: 5'-GCAACAGAUUGUGUUCUUA-3', reverse 5'-UAAGAACAUCUGUUUGC-3'. Nonspecific negative control primers (forward 5'-UUCUCCGAACGUGUCACGUTT-3', reverse 5'-ACGUGACACGUUCGGAGAATT-3') were purchased from Sigma-Aldrich (St. Louis, USA).

RNA isolation and real-time quantitative PCR. Total RNAs were extracted from transfected cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and were reverse-transcribed into cDNA using a ReverTra Ace qPCR Kit (Toyobo, Osaka, Japan). Real-time PCR was performed using an UltraSYBR Mixture (CWBIO). The sequences of the sense and antisense primers were as follows: $\beta 6$ integrin forward: 5'-AGGATAGTTCTGTTTCCTGC-3', and reverse: 5'-ATCATAGGAATATTTGGAGG-3'; GAPDH: 5'-AACGGATTTGGTCGTATTGGG-3', and reverse 5'-CCTGGAAGATGGTGATGGGAT-3'; MMP-2 forward: 5'-TGATCTTGACCAGAATACCATCGA-3', and reverse: 5'-GGCTTGCGAGGGAAGAAGTT-3'; MMP-3 forward: 5'-CGGTTCCGCCTGTCTCAAG-3' and reverse: 5'-CGCCAAAAGTGCCTGTCTT-3'; MMP-9 forward: 5'-ACCTCGAACTTTGACAGCGAC-3', and reverse: 5'-GAGGAATGATCTAAGCCCAGC-3'. Relative gene expression levels were normalized to GAPDH as a control.

Western blotting. After 48 hours of transfection, cells were subjected to protein extraction using cell lysis buffer containing 1% protease inhibitors. Protein concentrations of the homogenized lysates were measured using a BCA protein assay kit (Sangon, Shanghai, China). Aliquots containing 30 μ g of protein were separated by 10% SDS-PAGE and then transferred to PVDF membranes (Millipore, Billerica, MA, USA). Membranes were incubated overnight at 4 °C with the following primary antibodies: mouse monoclonal antibody against integrin $\beta 6$ (1:1000; Millipore, CA, USA), mouse monoclonal antibody against Rac1 (1:300; Abcam, Hong Kong), or anti- β -actin (1:1000; ZSGB-Bio, Beijing, China), followed by secondary antibodies (1:2000; goat-anti rabbit or mouse IgG, ZSGB-Bio) conjugated to peroxidase for 1 hour at room temperature. After washing, immunoreactivity was visualized using an enhanced chemiluminescence kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions.

Immunohistochemistry (IHC). IHC was performed using paraffin-embedded tissue sections. 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 in a stainless steel pressure cooker). Endogenous peroxidase was blocked using a 3% hydrogen peroxide solution. The section was incubated with the blocking goat serum for 15 min and then immunostained with rabbit antibody against $\beta 6$ (dilution 1:500) at 4 °C 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.

Evaluation of immunohistochemical staining. Immunohistochemical staining was independently evaluated by two experienced pathologists in a blinded manner. Staining was semi-quantitatively scored based on both the staining intensity (0, negative; 1, weak; 2, moderate; 3, strong) and the percentage of positively stained cells (0, 0%; 1, 1–25%; 2, 26–50%; 3, 51–75%; 4, 76–100%). Both scores for each specimen were then combined to obtain the final score of $\beta 6$ expression. The cut-off point for the sum of the scores was defined as follows: 0–2, low expression; 3–7, high expression. The appropriateness of the cut-off point was validated by ROC analysis.

Cell viability assays. A total number of 2500 cells was seeded in 96-well plates in triplicate wells and cultured for the indicated times. Cell viability 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. Each time point was replicated in three wells, and the experiment was independently performed at least three times.

Cell proliferation assays. Cell proliferation assays were performed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). A total number of 2500 cells was seeded in 96-well plates. Then, 24 hours after $\beta 6$ silencing or overexpression, MTT at 5 mg/ml was added and incubated for 4 hours. Finally, the medium was aspirated, DMSO was added, and the absorbance was determined at 490 nm. Each time point was repeated in three wells, and the experiment was independently performed at least three times.

Wound-healing assays. The monolayer wound-healing assay was used to assess cell migration. A total of 5×10^5 cells were seeded in 6-well plates, incubated overnight, and then pretreated with $\beta 6$ siRNA or the $\beta 6$ overexpression plasmid. After achieving 90% confluency, the cell monolayer was scratched with a sterile pipette tip, the floating cells were removed with PBS, and the cells were cultured for 24 hours. Photographic images were taken at 0 and 24 hours along the scrape line using a microscope. The results are expressed as the actual wound closure distance.

Transwell assays for cell migration and invasion. Tumor cell migration assays were analyzed in 24-well Boyden chambers with 8- μ m pore size polycarbonate membranes (Costar, Acton, USA). For invasion assays, the membranes were precoated with 50 μ g Matrigel (BD Biosciences, San Diego, USA) to simulate matrix barriers. Then, 1×10^5 cells were resuspended in 200 μ l serum-free medium and seeded into the upper chamber, and the lower compartments were filled with 600 μ l medium with 10% FBS. After incubation (incubation time for migration is 10–12 hours and for invasion is 24 hours), the upper surface of the Transwell membrane was wiped gently with a cotton swab to remove the non-migrating cells. The membranes were fixed with methanol and stained with 0.1% crystal violet for twenty minutes. The stained cells were counted under a light microscope at $\times 200$ magnification in at least five fields. NSC23766 (Calbiochem, San Diego, USA), a specific Rac1 inhibitor, was used to inhibit Rac1 activity in several experiments.

MMP activity assays. A total of 2×10^5 cells were cultured in a six-well culture plate and then treated with siNC, si $\beta 6$, mock plasmid or $\beta 6$ overexpression plasmid for 24 hours. The levels of secreted MMP-9 in the culture supernatant were determined using an enzyme-linked immunosorbent assay (ELISA) following the manufacturer's ELISA kit guidelines (R&D). Samples were assayed in triplicate and calibrated against a standard curve.

Immunofluorescence for F-actin staining. To observe the effect of integrin $\beta 6$ on F-actin, cholangiocarcinoma cells were seeded (1×10^4) on the cover slip and cultured overnight. Twenty-four hours after transfection, cells were fixed, permeabilized and then stained with tetramethylrhodamine (TRITC)-conjugated phalloidin (Sigma–Aldrich, St. Louis, USA) for 1 hour. Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI) (Beyotime) for 5 min. The results were analyzed with confocal laser microscopy (Carl Zeiss, LSM780, Oberkochen, Germany) and are expressed as the % decrease in F-actin = [(F-actin in untreated cells–F-actin in siNC or si $\beta 6$ treated cells)/F-actin in untreated cells] $\times 100$.

Rac1 activity assay. The levels of the active GTP-bound form of Rac1 were determined with p21-activated kinase (PAK)-GST protein beads (Cytoskeleton). Cell lysate was prepared as previously described⁴². The lysate was incubated with 20 μ g of PAK-GST protein beads for 30 min at 4 °C. Proteins on beads and in total cell lysates were assessed by SDS-PAGE followed by immunoblotting with a Rac1-antibody.

Xenograft tumor 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. Cultured RBE cells were infected with a lentiviral vector LV-negative control, LV-si $\beta 6$ or LV- $\beta 6$ (Genechem, Shanghai) using a standard protocol. To evaluate tumor growth *in vivo*, 1×10^7 RBE cells were injected subcutaneously into the flanks of nude mice. The tumor size was measured every three days, and the tumor volume was calculated as follows: longer diameter \times shorter diameter² $\times 0.5$. All mice were sacrificed after 22 days. Subcutaneous tumor grafts were excised and weighed. Then, the tumors were processed for standard IHC analysis. All animal studies were conducted using a protocol approved by the Institutional Animal Care and Use Committee of the School of Medicine, Shandong University (Jinan, Shandong, China).

Statistical analysis. The associations between $\beta 6$ expression and clinicopathological parameters were analyzed by the chi-square test and Fisher's exact test. Quantitative data are presented as the mean \pm SD. The statistical significance was determined by two-tailed paired Student's t-test in two groups and one-way ANOVA in multiple groups. Receiver operating characteristic (ROC) curve analysis was performed to assess the diagnostic value of $\beta 6$ in cholangiocarcinoma. All statistical analyses were performed using SPSS 18.0 software (SPSS Inc., Chicago, USA), and a *P* value < 0.05 was considered statistically significant.

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Author Contributions

J.N. was responsible for designing the study and critical review of manuscript; Z.L. and S.B. were responsible for performing the study, literature research and writing the manuscript; B.L. and X.Z. were responsible for data acquisition and analysis; L.S., Y.L. and R.F. were responsible for critical review of the manuscript. All authors approved the final version of the manuscript.

Additional Information

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