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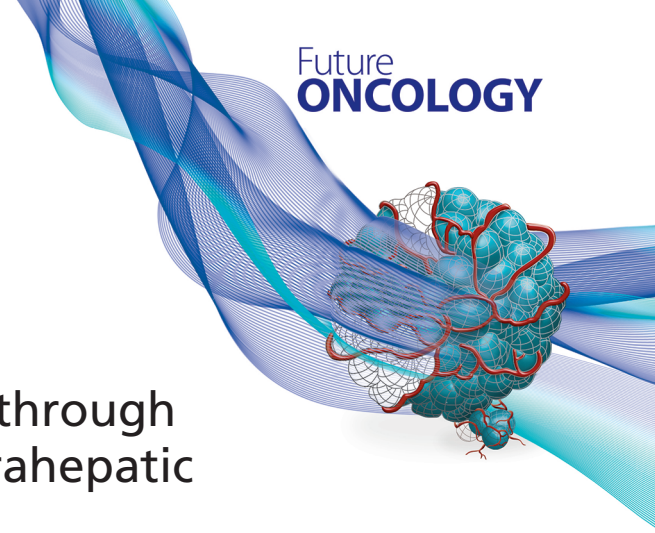

S100A11 promotes *TGF-β1*-induced epithelial–mesenchymal transition through *SMAD2/3* signaling pathway in intrahepatic cholangiocarcinoma

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Aim: Our previous study found S100A11 was significantly raised in intrahepatic cholangiocarcinoma cells, but the relationship between S100A11 and intrahepatic cholangiocarcinoma remains unclear. **Methods:** We investigated the effect of silencing S100A11 on *TGF-β1*-induced epithelial–mesenchymal transition (EMT), cell migration and invasion. **Results:** Our results demonstrated silencing S100A11 inhibited *TGF-β1*-induced cell migration, invasion and EMT, expression of EMT markers E-cadherin, N-cadherin, β-catenin, vimentin, Slug and Snail was reversed. Furthermore, *TGF-β1*-induced *p-SMAD2* and *3* were also inhibited due to low S100A11 expression. **Conclusion:** Our present study indicated that S100A11 promotes EMT through accumulation of *TGF-β1* expression, and *TGF-β1*-induced upregulation of *p-SMAD2* and *3*.

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Keywords: EMT • intrahepatic cholangiocarcinoma • invasion

Intrahepatic cholangiocarcinoma (ICC) is the second most malignant primary liver cancer, which originated in the intrahepatic bile duct epithelium. Over the past 20 years, the incidence of ICC has increased worldwide year by year [1,2], but there is still no progress in the treatment of ICC, even the early surgical treatment remains unsatisfactory with 5-year survival rate being still less than 10% [3,4]. The main reason for this rigorous situation is due to the lack of early diagnosis methods, lymphatic metastases and insensitivity to traditional treatments such as chemotherapy and radiotherapy [4,5]. Molecular biomarker able to diagnose ICC at an early stage is the key to future treatment for ICC.

S100 protein is a kind of acidic protein with relative molecular weight of 9–14 kDa, which is the largest subclass of calcium binding protein family [6]. S100A11, also known as S100C, is an important member of the S100 family [7], whose main function is to transduce calcium-dependent cell signals and to regulate cell cycle, cell differentiation and extracellular matrix secretion [8]. Emerging evidence has revealed that S100A11 is highly expressed in numerous cancers, including papillary thyroid carcinoma [8], colon [9], breast cancer [10], cholangiocarcinoma and non-small-cell lung cancer [11]. High expression of S100A11 is closely related to the malignant proliferation, distant metastasis and poor prognosis such as ovarian cancer [12], lung cancer [13] and renal cancer [14].

Epithelial–mesenchymal transition (EMT) is a cell process in which mature epithelial cells exhibit the appearance and characteristics of mesenchymal cells [15]. Cells during EMT mainly contain the following features: loss of cell–cell adhesion, acquisition of mesenchymal protein expression and gain of invasive characteristics [16]. Studies have shown that EMT is closely involved in the invasion of ovarian [17] and liver cancer [18–20]. Interestingly, recent study has confirmed that S100A11 serves a role in promoting malignant invasion of ovarian cancer through EMT [12].

TGF-β1 is a functionally complicated cytokine that regulates series of cellular functions, including differentiation, cell proliferation, apoptosis and adhesion [21]. *TGF-β1* is one of the most important induction factors to regulate EMT [22,23]. During *TGF-β1*-induced EMT, *TGF-β1* binds to the *TGF-β* type II receptor, then induces the

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Table 1. Sequences of three siRNAs.

siRNA sequence number		Nucleotide sequence(5′-3′)
S100A11-si-1	Sense	CUUCAUGAAUACAGAAUUAdTdT
	Antisense	UAGUUCUGUAUUCAUGAAGdTdT
S100A11-si-2	Sense	GGUUAUAACUACACUCUCUdTdT
	Antisense	AGAGAGUGUAGUUAUAACCdTdT
S100A11-si-3	Sense	CACCUGCCAAUAGUAAUAAdTdT
	Antisense	UUAUUACUAUUGGCAGGUGdTdT

recruitment and phosphorylation of the type I *TGF-β* receptor. The activated *TGF-β* type I receptor subsequently induces the recruitment and phosphorylation of the receptor-regulated SMADs, *SMAD2* and *3* to form heteromeric complexes with the common mediator SMAD4 [22]. The SMAD complexes transmit the signals of *TGF-β* from cell surface to nucleus and bind to the target genes to exhibit the regulated role [23]. Additionally, it is reported that high expression of *TGF-β1* is correlated with increased tumorigenicity and poor prognosis of ICC [24].

In this present study, we investigated the effect of S100A11 on *TGF-β1*-induced EMT, cell migration and invasion, and the underlying molecular mechanism was also explored.

Materials & methods

Cell lines & main reagents

The human cholangiocarcinoma cell line, RBE was obtained from the Chinese Scientific Academy. Three pairs of S100A11-siRNA were designed according to the S100A11 gene sequence and synthesized chemically by Biotend (Shanghai, China). The nucleotide sequence of S100A11-siRNA was shown in Table 1. Scrambled nonspecific siRNA was designed as a negative control group (Biotend), the sequence was 5′-UUC UCC GAA CGU GUC ACG UTT-3′.

Cell culture & transfection

RBE cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin–streptomycin (Hyclone, UT, USA), cultivated in 37°C incubator with 5% CO_2. Cells were planted in a six-well plate at a density of 1×10^5/well. When grown to 60%, the mixture of siRNA (50 nM siRNA) and Lipofectamine 2000 (Invitrogen, CA, USA) was added to cells according to the manufacturer's protocol. After 48 h, the efficiency of siRNAs was tested by western blot.

Establishment of *TGF-β1*-induced EMT model

TGF-β1 (PeproTech, NJ, USA) was used to induce EMT in RBE cells. Cells were seeded in six-well plates and incubated for 24 h, then treated with *TGF-β1* (0, 5, 10 and 20 ng/ml), cultivated for 48 h in 37°C incubator with 5% CO_2. Then, we used western blot to detect the protein expression of EMT markers and screened out the best induction concentration of *TGF-β1*. The proteins of N-cadherin and Snail were used to evaluate the induced effect.

Western blot

SDS-PAGE electrophoresis was used to separate equal amount of protein (30 μg) on 10 or 15% gel. The proteins were subsequently transferred to polyvinylidene fluoride (Millipore, CA, USA), and the membranes were blocked in 5% bovine serum albumin (BSA) for 1 h at room temperature, then incubated with primary antibodies at 4°C overnight. The details of primary antibodies are shown in Table 2. β-actin (Kangcheng, Shanghai, China) was used as an internal control. After 12 h, the membranes were washed three-times with tris buffered saline tween (TBST), incubated with secondary antibody (Kangcheng) for 1 h at room temperature. Finally, enhanced chemiluminescent substrate was used to detect protein bands and photos were analyzed by Image Lab software (Bio-Rad, Ontario, Canada).

Cell invasion & migration assays

Transwell assay was used to detect the invasion and migration of RBE cells. The experiment was divided into four groups: negative group (cells transfected with scrambled nonspecific siRNA); *TGF-β1* group (cells treated

Table 2. Details of primary antibodies.

Name	Producer	Antibody number	Dilution ratio
N-cadherin	Abcam, Cambridge, UK	76057	1:1000
E-cadherin	Abcam, Cambridge, UK	40772	1:1000
β-catenin	CST, Hochschule Darmstadt, Germany	8480	1:1000
Vimentin	CST, Hochschule Darmstadt, Germany	5741	1:1000
Slug	CST, Hochschule Darmstadt, Germany	9585	1:500
Snail	CST, Hochschule Darmstadt, Germany	3879	1:500
TGF-β1	Abcam, Cambridge, UK	92486	1:1000
p-SMAD2	CST, Hochschule Darmstadt, Germany	3180	1:1000
SMAD2	CST, Hochschule Darmstadt, Germany	5339	1:1000
p-SMAD3	CST, Hochschule Darmstadt, Germany	9520	1:1000
SMAD3	CST, Hochschule Darmstadt, Germany	9523	1:1000
S100A11	Abcam, Cambridge, UK	180593	1:1000

with *TGF-β1*); S100A11-siRNA (A11-siRNA) group (cells transfected with S100A11-siRNA sequence); and *TGF-β1*+A11-siRNA group (cells treated with *TGF-β1* and transfected with S100A11-siRNA sequence). For cell migration assay, cells transfected with S100A11-siRNA for 24 h or not were digested and resuspended with serum-free medium, then 200 µl cell suspension was put into the upper chamber (for cell invasion assay, 50 µl precooled matrigel (Becton-Dickinson, NJ, USA) should be coated on the surface of the upper chamber before putting 200 µl cell suspension into the upper chamber), and 600 µl complete medium containing 10% fetal bovine serum was added with or without *TGF-β1* to the lower chamber. After 24 h for migration or 48 h for invasion, the transwell chambers were taken out to be fixed with paraformaldehyde solution for 30 min, then dyed with Giemsa (Sigma-Aldrich, Darmstadt, Germany) for 20 min, inverted microscope (DMI300B; Leica Microsystems GmbH, Wetzlar, Germany) was used to observe the migrated and invaded cells.

Statistical analysis

SPSS20.0 was used to analyze the data, the differences between groups were analyzed by one-way analysis of variance or Student's t-test. $p < 0.05$ was considered to indicate a statistically significant difference. GraphPad Prism 5.0 software was adopted to dispose all the photographs. Three independent and repetitive experiments were conducted in each of our assay and data are presented as means ± standard deviation.

Results

The expression of S100A11 in clinical tissue specimens & the transfection efficiency of siRNA in RBE cells

First, we analyzed the published microarray datasets [25] and confirmed that the level of S100A11 was significantly upregulated in ICC tissues compared with both normal tissues (GEO/GSE32879, $p = 0.000$) and hepatocellular carcinoma (HCC; GEO/GSE15765, $p = 0.000$) (Figure 1A).

As our previous study demonstrated [25], the expression of S100A11 was increased in RBE cells, we therefore used RBE cells to conduct the whole experiment. Western blot was used to detect the efficiency of siRNA transfection in RBE cells, and the result showed that S100A11 expression was dramatically reduced after transfected with S100A11-siRNA-2 sequence (Figure 1B). For comprehensive consideration, we picked S100A11-siRNA-2 sequence to carry on the subsequent experiments.

TGF-β1 induces EMT in RBE cells

It was reported that *TGF-β1* was an important inducer during EMT process and closely related to migration and invasion of several tumors [22]. To confirm this hypothesis in ICC, RBE cells were treated with 0, 5, 10 and 20 ng/ml *TGF-β1* for 48 h, the protein expression of EMT markers N-cadherin and Snail was detected by western blot, showing a dose-dependent increase in N-cadherin and Snail protein expression and a significant increase when treated with 10 ng/ml *TGF-β1* (Figure 2A). In this experiment, we detected the expression of N-cadherin and Snail just to confirm whether *TGF-β1* initiated EMT in RBE cells and to verify the best concentration to induce EMT, the expression of other EMT markers after treatment with *TGF-β1* will be detected in later experiments.

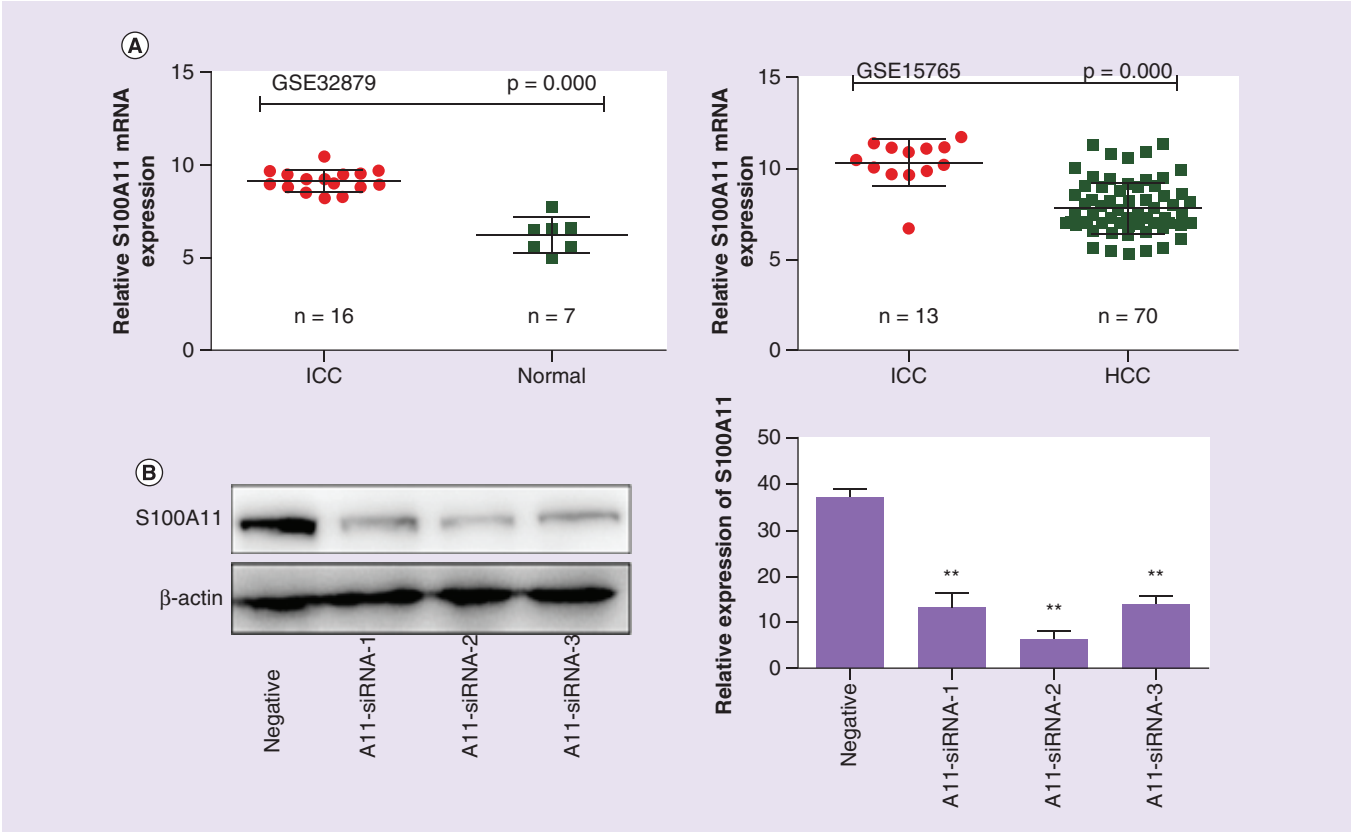

Figure 1. The expression of S100A11 in clinical tissue specimens and the transfection efficiency of siRNA in RBE cells. (A) Relative expression of S100A11 mRNA in ICC tissues was higher than normal and HCC tissues. **(B)** Western blot revealed S100A11 expression in RBE cells was significantly lower in S100A11-si-2 group.
**p < 0.01 versus negative group.
HCC: Hepatocellular carcinoma; ICC: Intrahepatic cholangiocarcinoma.

Therefore, *TGF-β1* at a concentration of 10 ng/ml was used to induce EMT in our experiment. Simultaneously, RBE cells underwent morphological changes, and gained a fibroblast-like appearance and elongated morphology when treated with 10 ng/ml *TGF-β1* (Figure 2B). Collectively, these results confirmed that *TGF-β1* initiated EMT in RBE cells.

Effect of S100A11 on EMT markers & *TGF-β1*

Related literature had confirmed that S100A11 can promote tumor invasion through EMT [12], but little was known about how S100A11 regulated EMT in ICC. To solve this doubt, western blot was used to investigate the effect of S100A11 on EMT in RBE cells. The result revealed that the expression of N-cadherin, β-catenin, vimentin, Slug and Snail was decreased and epithelial marker E-cadherin was increased when silencing S100A11. Taking into account the dual role of β-catenin in EMT, it cannot only suppress EMT through promoting cell adhesion, but facilitate EMT via regulating Snail activity [26]. In view of this mechanism, we can comprehend why β-catenin positively associated with EMT in our research. Moreover, significantly decreased TGF-β1 protein expression was observed after silencing S100A11 in RBE cells (Figure 3).

Silencing S100A11 suppresses *TGF-β1*-induced EMT in RBE cells

The results showed that RBE cells in the A11-siRNA group exhibited classical epithelial morphology, while cells in the *TGF-β1* group exhibited a fibroblast-like appearance and mesenchymal morphology, however, cells in *TGF-β1*+A11-siRNA group seemed to change little compared with A11-siRNA group, and still exhibited epithelial morphology without changing to an elongated and fibroblast-like phenotype (Figure 4A). The results of western blot demonstrated the expression of N-cadherin, β-catenin, vimentin, Slug and Snail was significantly decreased and

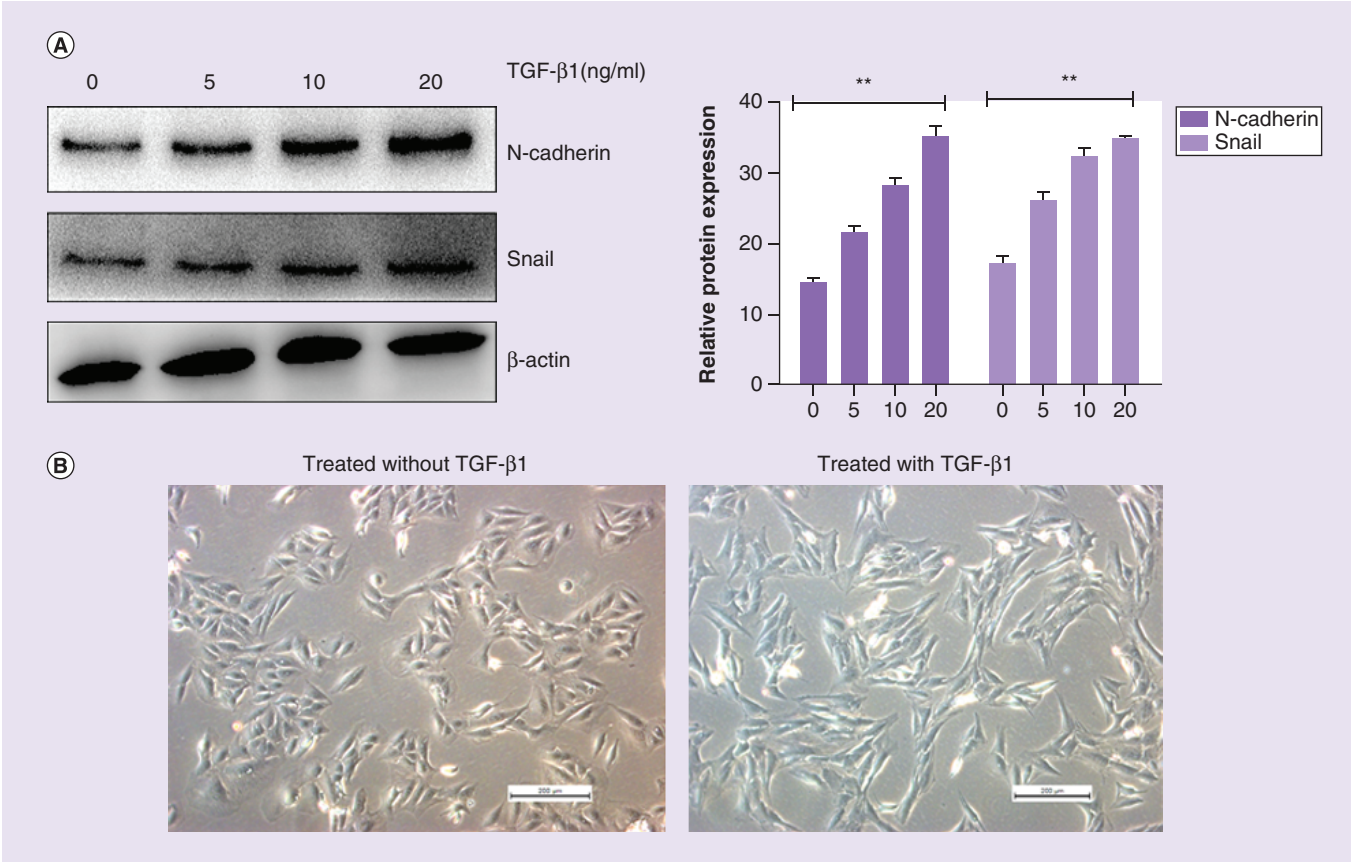

Figure 2. *TGF-β1* induced epithelial–mesenchymal transition in RBE cells. (A) A dose-dependent increase in N-cadherin and Snail protein expression was observed in RBE cells when treated with *TGF-β1*. **(B)** Representative images of morphological changes in RBE cells treated with or without 10 ng/ml *TGF-β1*, showing a fibroblast-like appearance and elongated morphology in *TGF-β1* group. At 100× magnification, scale bar = 200 μm.
**p < 0.01 versus RBE cells treated with 0 ng/ml TGF-β1.

E-cadherin was increased in *TGF-β1*+A11-siRNA group compared with *TGF-β1* group, no significant difference in these proteins' expression was observed between *TGF-β1*+A11-siRNA and A11-siRNA groups. In other words, *TGF-β1*-induced EMT was inhibited by silencing S100A11 in RBE cells (Figure 4B).

Silencing S100A11 inhibits *TGF-β1*-induced *SMAD2 /3* phosphorylation in RBE cells

During *TGF-β1* induced EMT process, phosphorylation of *SMAD2* and *3* was a critical step in the initiation of *TGF-β1* signal transduction [23]. Therefore, the effect of S100A11 on SMAD expression was investigated. As presented in Figure 4C, *p-SMAD2* and *3* expression was significantly decreased in *TGF-β1*+A11-siRNA group compared with *TGF-β1* group, but no significant difference in total *SMAD2* and *3* protein expression was observed between cell groups. And there was no significant difference in *p-SMAD2* and *3* expression between the *TGF-β1*+A11-siRNA and A11-siRNA groups. Collectively, these results demonstrated that silencing S100A11 decreased *TGF-β1*-induced *SMAD2* and *3* phosphorylation in RBE cells.

Silencing S100A11 suppresses *TGF-β1*-induced migration & invasion in RBE cells

Additionally, we investigated the effects of knockdown of S100A11 on *TGF-β1*-induced invasion and migration in RBE cells. As presented in Figure 5A & B, the number of migratory and invasive cells in *TGF-β1*+A11-siRNA group was significantly decreased compared with *TGF-β1* group. These results demonstrate that treatment with *TGF-β1* was able to promote RBE cells invasion and migration, and these effects were weakened greatly after silencing S100A11, indicating that low expression of S100A11 inhibited *TGF-β1*-induced cell invasion and migration.

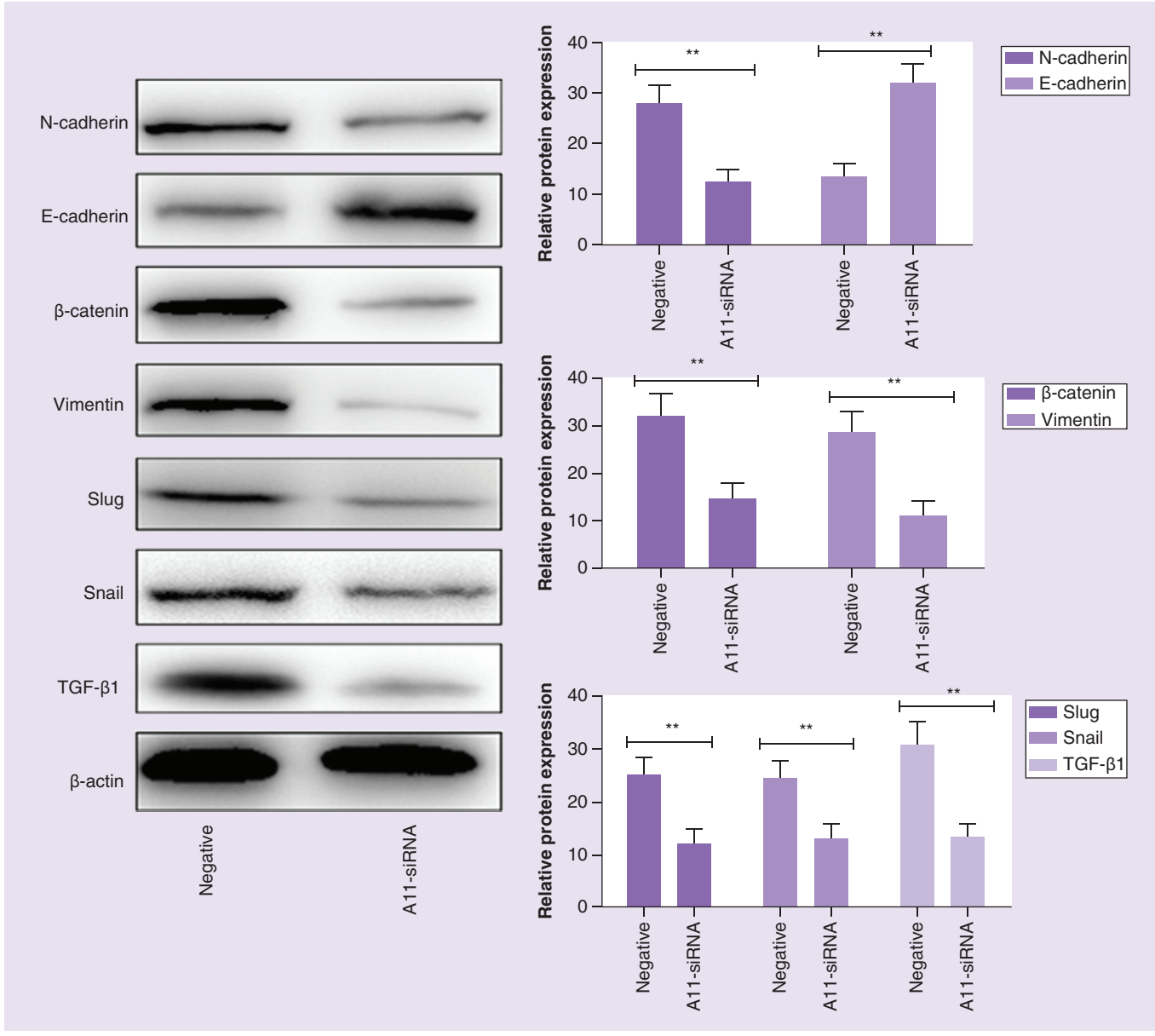

Figure 3. Silencing S100A11 on epithelial–mesenchymal transition markers and *TGF-β1*. The results of western blot showed the expression of N-cadherin, β-catenin, vimentin, Slug, Snail and *TGF-β1* was decreased and E-cadherin expression was increased in A11-siRNA group compared with negative group.
**$p < 0.01$.

Discussion

ICC is an extremely high malignant tumor with limited effective therapy and poor prognosis [4]. Lymph node metastasis at early stage is an important feature of ICC, however, the molecular mechanisms underlying metastasis remain unclear [25]. S100A11 is an important family member of S100 which closely related to tumor metastasis and EMT [12]. Our preliminary work found that S100A11 was highly expressed in ICC cells, but how S100A11 regulate ICC was poorly understood [25].

EMT serves a positive effect on tumor malignant development and promote tumor invasion in HCC cells [27]. Generally, EMT can be triggered by various external signals, among them, *TGF-β1* is the most important stimulus factor and plays an inducer role to promote EMT in HCC [28]. In our present research, it was observed that *TGF-β1* induced EMT in RBE cells. As presented in our result, RBE cells lost their classical epithelial morphology,

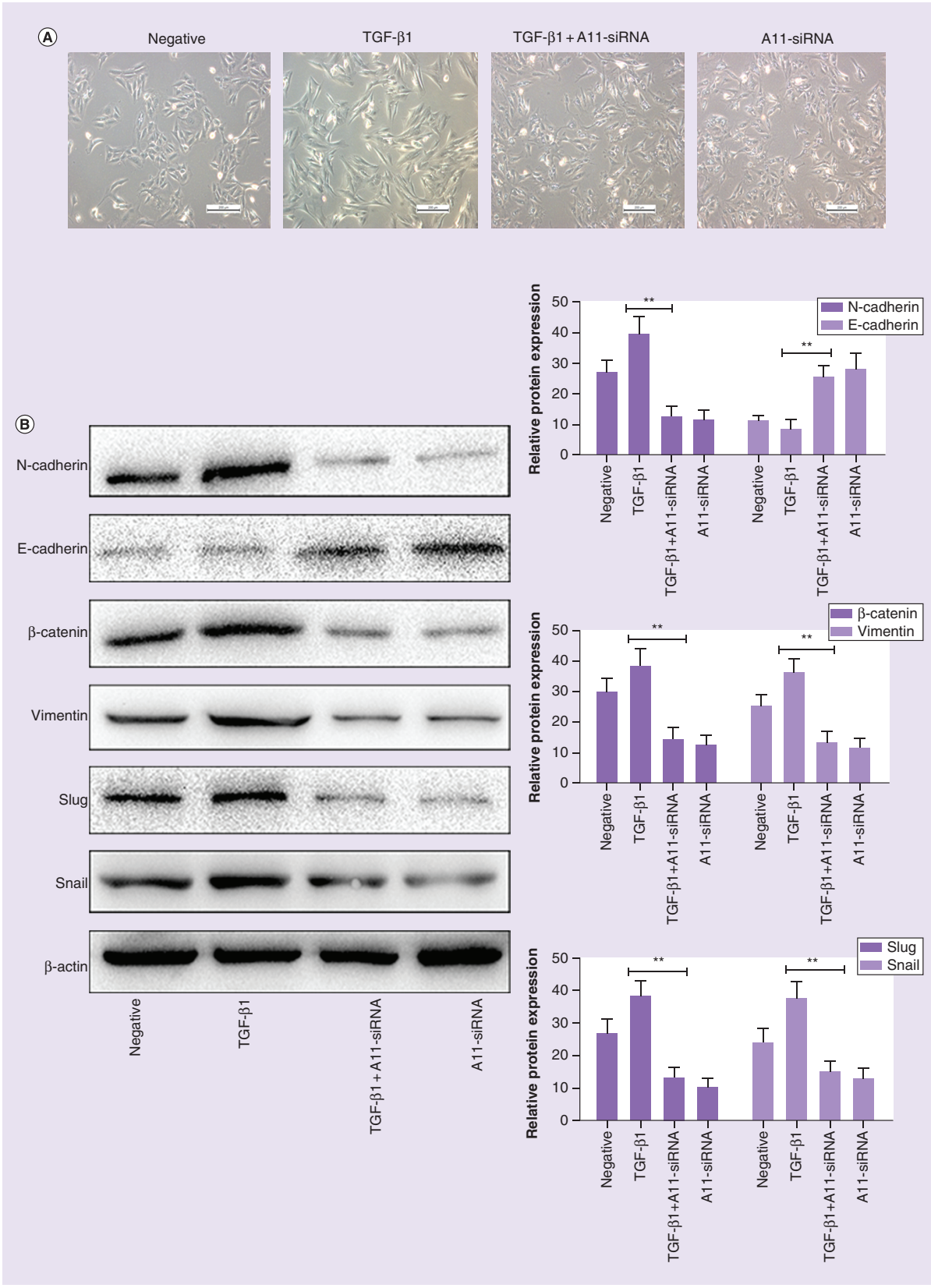

Figure 4. Silencing S100A11 suppresses *TGF-β1*-induced epithelial–mesenchymal transition and phosphorylation of *SMAD2* and *3*. (A) Representative images of RBE cells morphological changes in negative group, *TGF-β1*, *TGF-β1*+A11-siRNA and A11-siRNA groups. At 100× magnification, scale bar = 200 μm. **(B)** It was observed that expression of N-cadherin, β-catenin, vimentin, Slug and Snail was significantly decreased and E-cadherin was increased in the *TGF-β1*+A11-siRNA group. **(C)** Low expression of S100A11 inhibited the phosphorylation of *SMAD2* and *3* in *TGF-β1*+A11-siRNA group.
**$p < 0.01$ versus *TGF-β1* group.

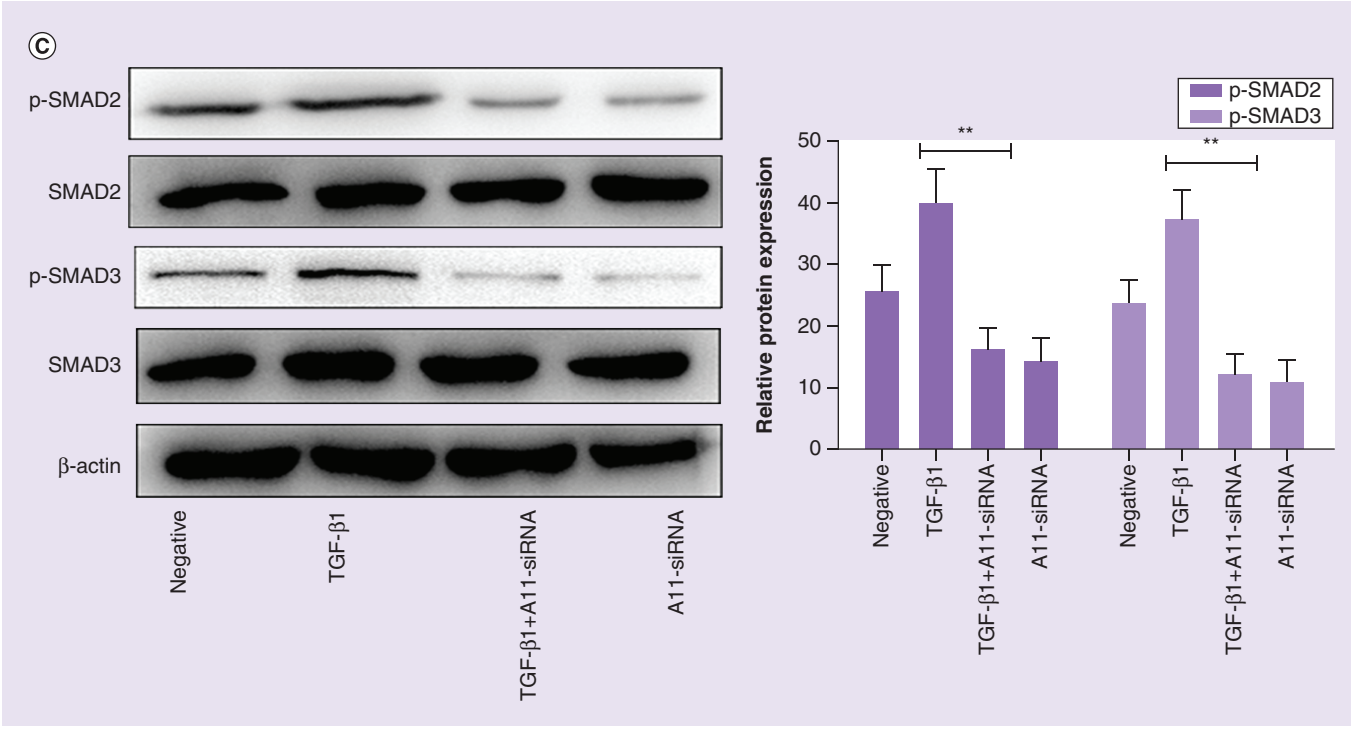

Figure 4. Silencing S100A11 suppresses *TGF-β1*-induced epithelial–mesenchymal transition and phosphorylation of *SMAD2* and *3* (cont.). (A) Representative images of RBE cells morphological changes in negative group, *TGF-β1*, *TGF-β1*+A11-siRNA and A11-siRNA groups. At 100× magnification, scale bar = 200 μm. **(B)** It was observed that expression of N-cadherin, β-catenin, vimentin, Slug and Snail was significantly decreased and E-cadherin was increased in the *TGF-β1*+A11-siRNA group. **(C)** Low expression of S100A11 inhibited the phosphorylation of *SMAD2* and *3* in *TGF-β1*+A11-siRNA group.
**$p < 0.01$ versus *TGF-β1* group.

and exhibited a mesenchymal morphology when treated with *TGF-β1*. Furthermore, protein expression of EMT makers N-cadherin and Snail significantly increased due to that RBE cells were sensitive to *TGF-β1*. To sum up, these data exactly verified our hypothesis that *TGF-β1* acted as an inducer during EMT process in ICC RBE cells.

S100A11 is a member of S100 family, and involves in aggressive development in several tumors [12]. Even some literature pointed out that S100A11 was able to speed up metastasis of ovarian cancer via EMT [12]. As our results demonstrated, silencing S100A11 decreased *TGF-β1* expression and inhibited *TGF-β1*-induced morphological changes in RBE cells and significantly suppressed *TGF-β1*-induced EMT, the protein expression of EMT markers such as N-cadherin and E-cadherin was reversed. These outcomes were consistent with the recent study that depletion of S100A4 (one member of S100 family) expression inhibited the *TGF-β* signaling pathway and weakened *TGF-β1*-mediated EMT in colorectal cancer cells [29]. We suspected that S100A11 as gene of promoting EMT can regulate *TGF-β1* expression and *TGF-β1*-induced EMT in ICC cells like the role of S100A4 in colorectal cancer cells. Furthermore, the increased number of invasive and migratory RBE cells by treatment with *TGF-β1* was significantly decreased to silence S100A11. Collectively, our results provided strong evidence that *TGF-β1*-induced EMT, cell invasion and migration were inhibited due to the low expression of S100A11 caused by siRNA.

Phosphorylation of *SMAD2* and *3* was the essential step in transducing *TGF-β* signals [23]. Based on this mechanism, we detected phosphorylation of *SMAD2* and *3* in RBE cells. It was observed that expression of *p-SMAD2* and *3* was increased after treatment with *TGF-β1* and this increase was significantly inhibited when silencing S100A11. Taken together, we speculated that low expression of S100A11 appeared to inhibit EMT via downregulation of *TGF-β1*-induced phosphorylation *SMAD2* and *3*, which was consistent with previous studies [30,31].

Several limitations to the present study should be noted. First, in our research, only ICC cell line RBE was included and we admitted that this was a deficiency of our research, but there were main three reasons to explain: our previous

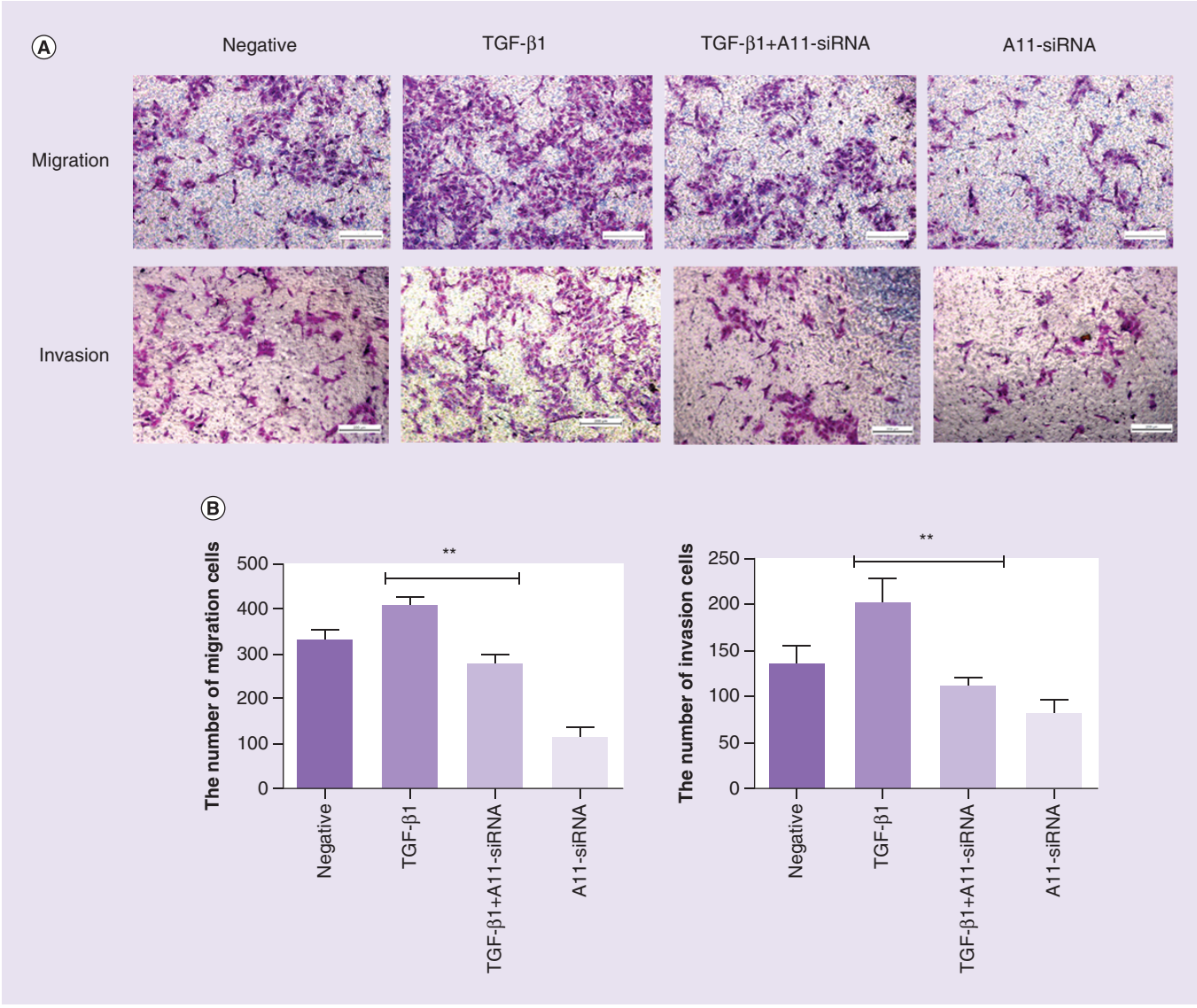

Figure 5. Silencing S100A11 suppresses *TGF-β1*-induced invasion and migration in RBE cells. (A & B) The number of migratory and invasive cells in the *TGF-β1*+A11-siRNA group was significantly decreased compared with *TGF-β1* group. At 100× magnification, scale bar = 200 μm.
**p < 0.01.

study had pointed out S100A11 was highly expressed in RBE cells, we further confirmed the regulatory function of S100A11 in RBE cells and our results were consistent with recent search [12]; we conducted three independent and repetitive experiments in each of our study, and we think our results were convincing and reliable; and there were some researches using only one cell line to prove their results [32,33]. Second, S100A11 was pointed out to be mainly involved in tumor metastasis and EMT, so in this present research, we only detected the relationship between S10A11 and *TGF-β*-induced EMT and migration and invasion, the experiments of cell proliferation, cell cycle, apoptosis and the underlying mechanism would be explored in the future. Third, we have not further studied the clinical implications of S100A11 in ICC patients.

Conclusion

Despite the acknowledged limitations, our research proves that silencing S100A11 inhibits *TGF-β1*-induced EMT, cells migration and invasion through attenuation of *TGF-β1* expression and inhibition of *TGF-β1*-induced *SMAD2* and *3* phosphorylation. It is worthy to consider blocking the S100A11-*TGF-β1-SMAD2/3* signaling pathway

combined with chemoradiotherapy to prevent metastasis in ICC clinical treatment. Further studies investigating serum S100A11 levels of ICC may be a better choice for S100A11 entering into clinical application.

Summary points

- S100A11 is highly expressed in intrahepatic cholangiocarcinoma tissues compared with both normal tissues and hepatocellular carcinoma tissues.
- Silencing S100A11 decreased *TGF-β1*-induced epithelial–mesenchymal transition through SMAD2/3 signaling pathway in intrahepatic cholangiocarcinoma cells.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

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