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STAT3 promotes migration and invasion of cholangiocarcinoma arising from choledochal cyst by transcriptionally inhibiting miR200c through the c-myb/MEK/ERK signaling pathway

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ABSTRACT

Signal transducer and activator of transcription 3 (STAT3) have been highlighted in cancer regulation. Its roles in Cholangiocarcinoma (CCA) arising from the choledochal cyst (CC) were unclear. Here, we attempted to elucidate the roles of STAT3 in CCA-CC and explore its mechanism. A total of 20 patients with CCA arising from CC, that underwent CC excision in the infant stage were included. The expressions of STAT3, miR200c and c-Myb in clinical samples were assessed by RT-qPCR and/or western blot. Their expression correlations in tumor tissues were evaluated by Pearson correlation analysis. Their roles in CCA cell migration and invasion were investigated by gene silence using siRNA or miRNA inhibitor mediated approach and MEK activator. The expression levels of EMT, metastasis and MEK/ERK pathway-related proteins were checked by western blot. The high expressions of STAT3 and c-Myb, and low expression of miR200c were detected in CCA samples. We defined the transcription inhibition of STAT3 in miR200c expression and the negative correlation between miR200c and c-Myb expression. Silence of STAT3 increased miR200c expression and retarded the migration and invasion of CCA cells, accompanied by decreased levels of Vimentin, N-cadherin, MMP2 and MMP9, and elevated expression of E-cadherin, resulting in inactivating MEK/ERK pathway. MiR200c inhibitor reversed the changes induced by STAT3 silence, which was restored by si-c-Myb. MEK activator significantly reversed the inactivation of the MEK/ERK pathway induced by si-STAT3+miR200c inhibitor+si-c-Myb. In summary, the silence of STAT3 suppressed metastasis and progression of CCA cells by regulating miR200c through the c-Myb mediated MEK/ERK pathway, suggesting STAT3 is the effective target for CCA arising from CC.

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Introduction

The choledochal cyst (CC) also served as biliary cysts, is usually a congenital condition and characterized by bile duct dilatation. Although it is a rare disease, CC is more common in Asian females than Western population. In addition, CC is a childhood disease with a 60% diagnostic rate in patients within 10 years old (1). Infant patients with CC are present with bile retention in bile ducts, finally leading to permanent liver damage with delayed treatment. Despite CC resection has been recommended to be the effective management for CC-induced infection and malignant transformation, cholangiocarcinoma (CCA) can be developed in the remaining biliary tract of patients after cholecystectomy.

CCA arising from CC presents a poor prognosis and high recurrence, which remains to be a challenge in the clinic. Much attention has been attracted to the genetic and epigenetic alterations underlying CCA development from CC. MicroRNAs (miRNAs) with variable expressions are associated with the oncogenesis and progression of cancers. MiR200 family is responsible for epithelial phenotype and is correlated with metastasis of cancer cells (2). It is reported that the silence of the miR200 family is associated with epithelial-to-mesenchymal transition (EMT) in the pathogenesis of CCA in CC (3). Inactivation of miR200c is associated with EMT induction with increased metastasis status in CCA cells (4). Besides, EMT is a dynamic process by which epithelial cells gradually adopt the structural and functional characteristics of mesenchymal cells, and it is closely linked to the malignant behaviors of CCA cells (5). However, the regulatory mechanism regarding miR200c has not been clarified in CCA arising from CC.

STAT3 is a transcription factor (TF), which is associated with aberrant proliferation and malignant transformation of tumor cells (6). The overexpression of STAT3 was determined in various cancers, such as breast cancer, leukemia and conventional CCA (7). Recent evidence has suggested that activated STAT3 signaling promoted the development of intrahepatic CCA (8). High glucose-induced upregulation of STAT3 to enhance CCA migration (9). Currently, the regulatory roles of STAT3 in CCA arising from CC were unknown.

C-Myb is a protooncogene, which is overexpressed in multiple cancers and accelerates the invasion and migration of tumor cells by enhancing EMT (10, 11). It is reported that c-Myb plays a regulatory role in MEK/ERK signa-

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ling involved in the proliferation and invasion of liver cancer cells (12). C-Myb was a functional target of miR200c (13, 14). Whether c-Myb regulated MEK/ERK pathway was involved in the tumor formation and progression of CCA in CC has been unknown.

Here, we investigated the regulatory role of STAT3 in miR200c expression and explored whether the intervention of STAT3 expression could affect the metastasis of CCA cells through c-Myb/MEK/ERK signaling.

Materials and Methods

Samples

Between 1999 February and 2019 February, 20 pairs of CCA and adjacent normal tissues were resected from patients with CCA arising from CC ranging from 18-60 years old (5 male and 15 female) with written informed consent. All the included patients underwent CC excision in the infant stage and had no chronic diseases and other tumors.

Cell culture and transfection

Three cholangiocarcinoma cell lines: TFK-1, FRH-0201 and QBC939 and a normal extrahepatic bile duct epithelial cell line (HEBEC) were ordered from Procell (Wuhan, China) and used in this study. All the cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (Gibco, USA) (15). Si-STAT3, miR200c inhibitor, miR200c mimics, si-c-Myb and negative controls were purchased (Sangon, China). Cell transfection was performed in compliance with the Manufacturer's Specification of Lipofectamine 3000 reagent (Thermo Fisher, USA) and cells without transfection were used as controls.

RT-qPCR analysis

Total RNA from tissues and cells was obtained with Trizol (Beyotime China), followed by cDNA synthesis using a commercial reagent kit (Beyotime China). The gene and miRNA expression profiles were analyzed by a real-time PCR system according to the previous description (3). MiRNA expression relative to U6 and gene expression relative to β-actin were calculated by the $2^{-\Delta\Delta CT}$ method. The intended primer sequences were listed in Table 1.

Western blot

Protein extraction was conducted with the application of RIPA (Beyotime China). The qualified protein determined by the BCA assay kit (Beyotime China) was separated by the Bis-Tris Gel system (Beyotime China). The proteins of interest were transferred to the PVDF membrane (Merck, USA). Primary antibodies (STAT3, MMP2, MMP9, E-cadherin, Vimentin, N-cadherin, MEK, p-MEK, ERK, p-ERK and GAPDH) were purchased (CST, USA) and diluted at 1:1000 by 5% blocking buffer and incubated with membranes overnight at 4°C. The second antibody incubation was performed at room temperature for 2 h. The blots were captured by the Chemiluminescent system and the proteins were quantitively analyzed by Image J software.

Dual-luciferase reporter assay

The wide type c-Myb luciferase plasmids containing the binding sequences for miR200c (c-Myb WT) and the mutant c-Myb plasmids containing the mutant binding site (c-Myb MUT) were synthesized by BGI (Beijing, China). C-Myb WT or c-Myb MUT plasmids and miR200c mimics/ miR-ctrl were co-transfected to FRH-0201 cells by Lipofectamine 3000 (Thermo Fisher, USA). After maintaining for 48 h, the relative luciferase activity was detected by a Microplate reader (Bio-rad, USA).

ChIP-PCR analysis

IP of RNA and proteins was performed in the FRH-0201 cell line following the previous description (16). Cells (2×10^7) were digested with 0.25% trypsin and collected for cell lysis. Cell lysates were centrifugated at 13000 rpm for 10 min. The supernatant was incubated with anti-STAT3 or IgG-coated protein C beads at 4°C for 24 h. The co-immunoprecipitated RNA was eluted from beads and detected by RT-qPCR assay.

Transwell assay

CCA cells at 1×10^5 /ml were seeded in the upper transwell inserts coated with or without Matrigel (Corning, USA) for invasion and migration assay, respectively. Cells in the upper chamber were maintained in serum-free media and those in the lower compartment were incubated in complete media with 10% FBS. After 24 h incubation, the media was removed and the cells in the upper chamber were wiped off with a pipette tip. The cells on the outer layer of the upper compartment were counted after staining with 0.1% crystal violet.

Statistical analysis

The quantitative data were presented as mean±SD and analyzed by GraphPad Prism and SPSS 25.0. The diffe-

Table 1. Primer sequences for PCR analysis.

Symbol	Primer sequence
miR200c	Forward: CCGGAATTCAGGGCTCACCAGGAAGTGTC, Reverse: CGCGTCGACGGCCCATTGTGTCCCTTAGT
STAT3	Forward: GGAGGAGTTGCAGCAAAAAG, Reverse: TGTGTTTGTGCCCAGAATGT
c-Myb	Forward: GAATAAAGGAGCTGGAGTTGCTC, Reverse: GTGCATCTAAGCCCGAGCTTTC
U6	Forward: GGAAGCTTGTCATCAATGGATATC, Reverse: TGATGACCCTTTTGGCTCCAAC
β-actin	Forward: CATTGTTACCAACTGGGACGACAT Reverse: GCCTCGGTGAGCAGCTTACA

rence between groups was processed by student t-test or one-way ANOVA. P<0.05 was considered significant.

Results

The expression of STAT3 in CCA tissues and its correlation with the expression of miRNA 200c, c-Myb and clinical features of patients

The expressions of STAT3, miR200c and c-Myb in 20 pairs of CCA tissues and normal counterparts were detected by RT-qPCR analysis. As illustrated in Fig 1A, STAT3 was remarkably overexpressed in CCA tissues, compared with adjacent normal counterparts (p<0.001). MiR200c was obviously under-expressed in CCA tissues, while c-Myb expression was raised in CCA tissues compared with adjacent normal counterparts (all p<0.001, Fig 1B and C). Correlation analysis indicated a negative correlation between the expression level of miR200c with that of STAT3 and c-Myb (Fig 1D and E). Additionally, patients were divided into two groups based on the median of STAT3 expression. Table 2 showed that there was a significant difference in the TNM stage and lymph node invasion between these two groups. Thus, we speculated that STAT3 expression was correlated with migration and invasion of CCA arising from CC by interacting with miR200c and c-Myb.

The expression levels of STAT3 and miR200c in CCA cells and their interaction

To explore the relationship between STAT3 and miR200c, the expressions of STAT3 and miR200c were first measured in TFK-1, QBC939, FRH-0201 and HEBEC cells. Results suggested that the expression of STAT3 was higher in CCA cells than in HEBEC cells, while miR200c exhibited a lower expression in CCA cells, especially for QBC939 and FRH-0201 cells (p<0.05, Fig 2A and B). The consistent STAT3 expression profile was determined by western blot (Fig 2C). QBC939 and FRH-0201 cells presented the highest STAT3 expression and lowest miR200c expression, which were selected for further experiments.

To further determine the regulatory interaction between STAT3 and miR200c, the STAT3 was knocked down in QBC939 and FRH-0201 cells by siRNA transfection. The efficiency of si-STAT3 transfection was checked by RT-qPCR and western blot (Fig 2D and E) and the expression of miR200c was significantly elevated by STAT3 knockdown (p<0.01, Fig 2F). STAT3-miR200c DNA interaction in the miR200c promoter was further confirmed by ChIP-PCR analysis (Fig 2G). Thus, we concluded that STAT3 repressed miR200c expression by binding to the miR200c promoter.

STAT3 knockdown inhibits the migration and invasion of CCA cells by regulating miR200c

As described in the previous studies, the high level of STAT3 was closely associated with the development, differentiation and migration of tumor cells (17). To explore

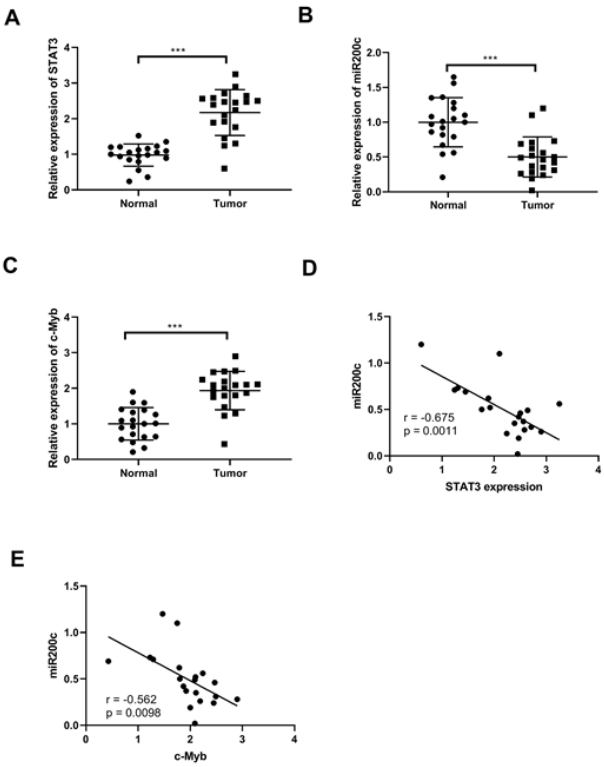

Figure 1. The expression of STAT3, miR200c, and c-Myb in CCA-CC tumor tissues and their expression correlation. The expressions of STAT3 (A), miR200c (B) and c-Myb (C) in 20 pairs of CCA tissues and normal counterparts were detected by RT-qPCR analysis. The expression correlation between miR200c with STAT3 (D) and c-Myb (E) was analyzed by Pearson correlation analysis. ***p<0.001.

Table 2. Correlation between STAT3 expression with clinical information.

Clinicopathological parameters	Total cases (n=20)	STAT expression		P value
		Low (n=9)	High(n=11)	
Age				0.964
>40	9	4	5	
<40	11	5	6	
Gender				0.795
female	15	7	8	
male	5	2	3	
TNM stage				0.024
I-II	6	5	1	
III-IV	14	4	10	
Lymph node invasion				0.005
Yes	13	3	10	
No	7	6	1	

the impact of STAT3 in cell migration and invasion, involved with miR200c expression, QBC939 and FRH-0201 cells were co-transfected with si-STAT3 and miR200c inhibitor, followed by detection of migration status and related protein expression via transwell assay and western blot. The results exhibited that compared with the control or si-CON group, the migration and invasion ability of FRH-0201 and QBC939 cells were reduced by STAT3 knockdown, which was abolished by miR200c inhibitor treatment ($p<0.05$, Fig 3A). Parallelly, the extracellular matrix remodeling markers (MMP2/9) and the mesenchymal markers (Vimentin and N-cadherin) in CCA cells were down-regulated, while the epithelial marker (E-cadherin) was up-regulated by STAT3 loss (all $p<0.05$). The changes elicited by si-STAT3 were abrogated by miR200c inhibitor treatment ($p<0.05$, Fig 3B).

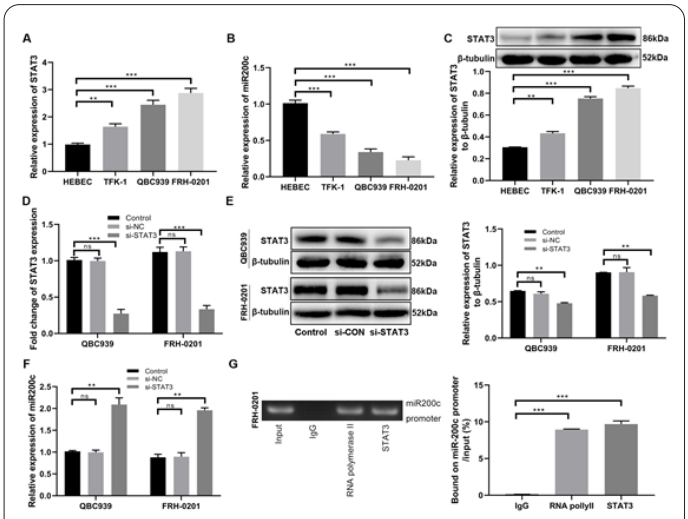

Figure 2. The relationship between STAT3 and miR200c. The expression of STAT3 (A) and miR200c (B) was detected in three CCA cell lines (TFK-1, QBC939, FRH-0201) and the normal intrahepatic bile duct epithelial cell line HEBEC. (C)STAT3 in CCA cells was also detected by western blot. The transfection efficiency was evaluated by RT-qPCR analysis (D) and western blot (E) after si-STAT3 transfection in QBC939 and FRH-0201 cells. (F) miR200c expression was evaluated by RT-qPCR analysis after si-STAT3 transfection. (G) Chip-PCR analysis was performed in FRH-0201 cells to confirm the transcriptional regulation of STAT3 on miR200c. Ns, no significant difference. *p<0.05, **p<0.01, ***p<0.001.

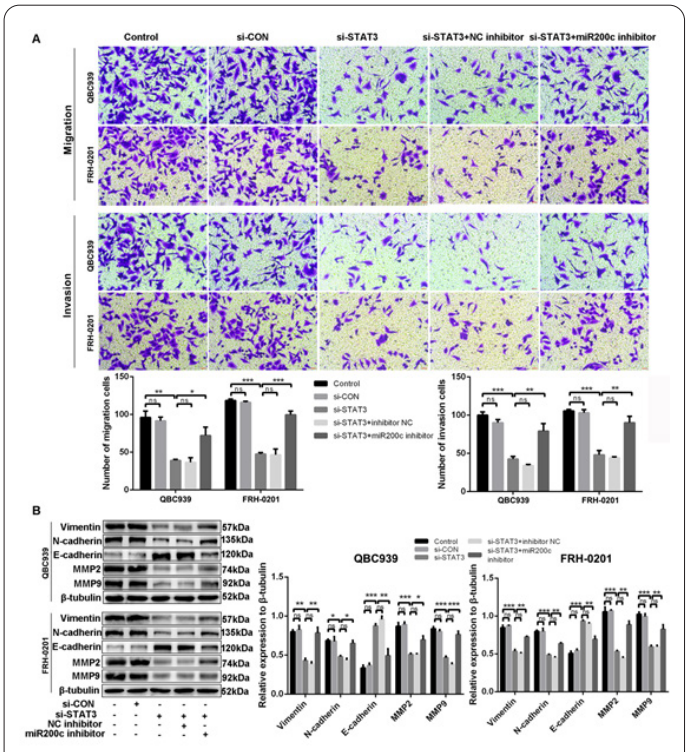

Figure 3. STAT3 silence reduced migration and invasion of CCA cells. (A)After si-STAT3 transfection, the migration and invasion status of QBC939 and FRH-0201 cells were detected by transwell assay. (B) EMT-related factors were analyzed by western blot. Ns, no significant difference. *p<0.05, **p<0.01, ***p<0.001.

STAT3 knockdown abrogates c-Myb mediated MEK/ERK signaling by up-regulating miR200c expression

C-Myb is a protooncogene that controlled the proliferation and invasion of liver cancer cells through the MEK/ERK pathway (12). C-Myb was found to be a functional target of miR200c in several types of tumor cells (13, 14). We determined the interaction between miR200c and c-Myb in FRH-0201 cells. The dual-luciferase report assay revealed that the luciferase activity declined in cells transfected with c-Myb WT plasmids containing c-Myb-miR200c binding sequences ($p<0.001$, Fig 4A). To further validate c-Myb-miR200c interaction, FRH-0201 cells were transfected with miR200c mimics. After checking the transfection efficiency by RT-PCR (Fig 4B), the

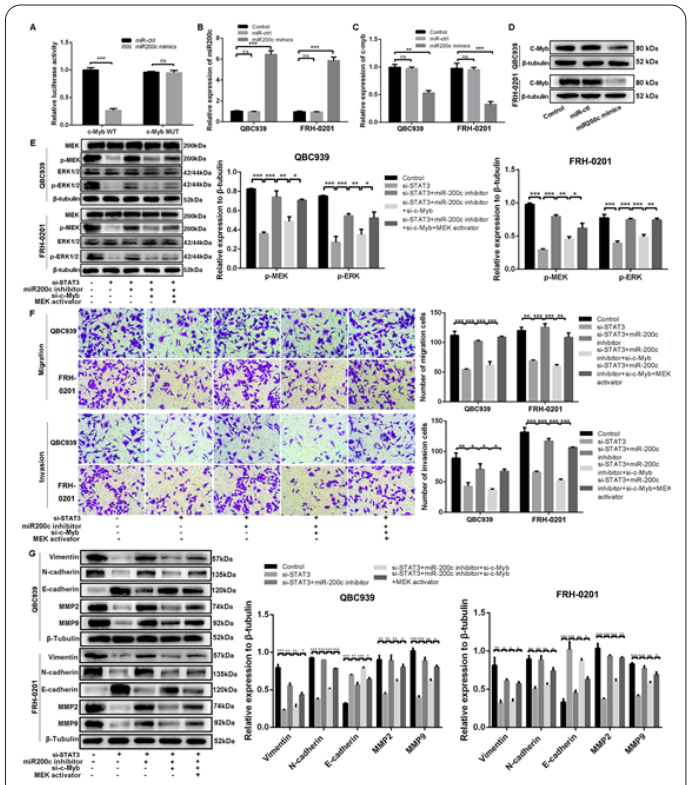

Figure 4. STAT3 silence reduced metastasis of CCA cells by inhibiting miR200c expression through c-Myb mediated MEK/ERK signaling. (A)The relationship between miR200c and c-Myb was determined by the dual-luciferase reporter assay. After QBC939 and FRH-0201 cells were transfected with miR200c mimics, the expression of miR200c (B) and c-Myb (C) was detected by RT-qPCR analysis. (D) The expression of c-Myb was detected by western blot after miR200c mimic transfection. (E) Western blot for MEK/ERK pathway-related proteins. (F) Transwell assay for migration and invasion of QBC939 and FRH-0201 cells. (G) Western blot for EMT-related protein expression. Ns, no significant difference. *p<0.05, **p<0.01, ***p<0.001.

c-Myb expression elicited by miR200c mimics was investigated by RT-PCR and western blot. Results indicated that the mRNA and protein expressions of c-Myb were pronouncedly reduced in FRH-0201 and QBC939 cells by miR200c mimic treatment, compared with Control or miR-ctrl group (all $p<0.01$, Fig 4C and D). All these above indicated that c-Myb was a target of miR200c in CCA cells.

Whether MEK/ERK signaling was involved in STAT3-mediated migration and invasion of CCA cells was explored. Western blot was executed to investigate the signaling pathway-related proteins. Fig 4E illustrated that the phosphorylated MEK and ERK levels declined in FRH-0201 and QBC939 cells by si-STAT3 treatment, which was reversed by miR200c inhibitor transfection (all $p<0.001$). Furthermore, the transwell assay revealed that si-STAT3 elicited the declined cell migration and invasion ability of both FRH-0201 and QBC939 cells (all $p<0.01$). The enhanced migration and invasion ability induced by the miR200c inhibitor was strikingly repressed by si-c-Myb (all $p <0.05$). The number of migrative and invasive cells was increased in the si-STAT3+miR200c inhibitor+si-c-Myb+MEK activator group compared with that in si-STAT3+miR200c inhibitor+si-c-Myb ($p<0.05$, Fig 4F). Concomitantly, the EMT markers (N-cadherin, vimentin) and MM2/9 were significantly declined, while E-cadherin was accumulated in cells transfected with si-STAT3, which was suppressed by co-transfected with miR200c inhibitor (all $p<0.05$). The changes of EMT-related proteins expression in the si-STAT3+miR200c inhibitor + si-c-Myb group was reversed by MEK activator addition in the si-STAT3+miR200c inhibitor+ si-c-Myb +MEK activator group ($p<0.05$, Fig 4G). Taken together, STAT3 loss relieved the invasion and migration of CCA cells through inhibiting c-Myb mediated MEK/ERK signaling by modulating miR200c expression.

Discussion

CC is a significant risk factor for CCA. CCA arising from CC is characteristic of aggressive metastasis and a high recurrent rate, which is associated with poor prognosis. The EMT process constitutes the key reason for tumor metastasis and this process is determined to be coordinately regulated by TFs. However, the TFs possess potential as the therapeutic target in CCA arising from CC, remains elusive. The previous study has shown that miR200c is a critical miRNA closely related to EMT in CCA arising from CC (3).

STATs belong to a class of TFs, which exert critical function in cell functions by mediating proliferation, apoptosis and angiogenesis. STAT3 as a member of the STAT family is overexpressed in a variety of tumor cells and tissues and plays a regulatory role in the progression and metastasis of cancers by regulating gene and miRNA expression (18). STAT3 has been supported to be a therapeutic target for cancers. It is reported that STAT3 suppressed the expression levels of pro-apoptosis-related genes in cancer cells (19).

In intrahepatic CCA, STAT3 is found to be overexpressed in tumor tissues, which promotes metastasis and poor prognosis of CCA (7). In this study, we determined the up-regulation of STAT3 expression in tumor tissues of CCA arising from CC, and high STAT3 level was closely related to the TNM stage and lymph node invasion. Our findings were in agreement with the previous study mentioned above. Additionally, Dokduang et al., suggested that STAT3 was inactivated in suppressed growth and increased apoptosis of human CCA cells mediated by Xanthohumol (20). Myricetin targeting STAT3 signaling inhibited metastasis of CCA cells by down-regulating metastasis-associated genes such as COX2 and MMP9 (21). FTY720 served as an immunosuppressant that elicited anti-metastasis of CCA cells by decreasing p-STAT3, N-cadherin, Vimentin and VEGF (22). To explicate the role of STAT3 in CCA arising from CC, STAT3 was knocked down in CCA cells by transfecting with siRNA. Results indicated that STAT3 silence inhibited migration and invasion of CCA cells, paralleled with the declined EMT markers (N-cadherin and vimentin) and metastasis biomarkers (MMP2/9). All these suggested that STAT3 overexpression could accelerate the metastasis of CCA in CC.

Furthermore, miR200c is underexpressed in tumor tissues of CCA in comparison with the non-tumor counterparts and its low expression presented a poor prognosis (18). MiR200c silence enhanced the invasion and migration of CCA cells (3). MiR200c is involved in the EMT process and is de-expressed in invasive cells of breast cancer (23). MiR200c is an EMT-associated miRNA, which was lowly expressed in scirrhous gastric cancers, and its overexpression abolished the EMT status of tumor cells (24). The transcriptional inhibition role of STAT3 on miR200c in CCA cells was determined in our study. Our data also indicated that the suppressed metastasis of CCA cells induced by si-STAT3 was reversed by miR200c knockdown, suggesting that STAT3 promoted metastasis of CCA cells by regulating miR200c expression. STAT3-miR200c axis could play a key role in CCA arising from CC.

Moreover, MEK/ERK signaling is an EMT-related pathway, which is activated in the metastasis of tumor cells (25-27). c-Myb is a proto-oncogene overexpressed in various cancers, CCA inclusive. The antagonistic interaction between miR200c and c-Myb was revealed in previous papers (13, 14), which was also determined by the dual-luciferase reporter assay in this study. All these above prompted us to explore whether STAT3 exerted pro-metastasis function in CCA through c-Myb mediated MEK/ERK signaling. The levels of p-MEK and p-ERK were determined when the expressions of STAT3, and/or miR200c and/or c-Myb were inhibited. Data in our study indicated that STAT3 knockdown significantly inhibited the expression of p-MEK and p-ERK, which was reversed by miR200c inhibition. The increased MEK and ERK phosphorylation following miR200c inhibition was further decreased by c-Myb knockdown. The altered STAT3/miR200c/c-Myb was involved with the changes in MEK/ERK pathway activation. Moreover, the invasive and migrative ability of CCA cells in the si-STAT3+miR200c inhibitor group were reduced by si-c-Myb in the si-STAT3+miR200c inhibitor + si-c-Myb group, accompanied by the up-regulation of E-cadherin and down-regulation of MMP2, MMP9, vimentin and N-cadherin. Thus, it is probably suggested that MEK/ERK signaling is involved in STAT3-mediated cell migration by targeting miR200c through the c-Myb-modulated MEK/ERK pathway.

In conclusion, STAT3 as a critical TF negatively regulated the expression of miR200c in tumor tissues of CCA

arising from CC and CCA cells. The diminished miR200c expression promoted the metastasis of CCA cells and activated MEK/ERK pathway by increasing c-Myb expression. Exogenous silence of STAT3 significantly alleviated the migration and invasion of CCA cells by targeting miR200c and inhibiting the c-Myb/ MEK/ERK pathway, which suggested a new effective therapeutic regimen for CCA in CC.

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

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Ethics approval

The study was approved by the Ethics Committee of Dongying People's Hospital and obeyed the principles of the Declaration of Helsinki.

Conflict of interest

The authors have declared that no competing interest exists.

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