European Review for Medical and Pharmacological Sciences 2020; 24: 8367-8376

LncRNA CASC2 inhibits cell proliferation, metastasis and EMT through miR-18a/SOCS5 axis in cholangiocarcinoma

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Abstract. – OBJECTIVE: Cholangiocarcinoma (CCA) is one of the tumors with high malignancy of the liver and bile system, whose development and prognosis mechanisms are still not clear. Here, a preliminary illustration was made on the expression and function of long non-coding RNA (lncRNA) CASC2 and the relevant mechanism of its function.

PATIENTS AND METHODS: Expression of CASC2 in CCA tissues and cells were examined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Cell proliferation ability was detected using colony formation and Cell Counting Kit-8 (CCK-8) assays while cell invasion and migration abilities were measured using transwell and Matrigel assays. Using bioinformatic analysis, underlying downstream molecules of CASC2 were predicted and by Dual-Luciferase assay and Western blot.

RESULTS: It was found that CASC2 was expressed at a significantly lower level in CCA tissues and cell lines. The overexpression of CASC2 inhibited QBC939 cell proliferation, invasion and migration when the knockdown of CASC2 accelerated HUCCT1 cell growth and metastasis. Besides, miR-18a was identified as a direct target for CASC2, and SOCS5 as target for miR-18a. Moreover, CASC2 functioned as a sponge of miR-18a to promote the SOCS5 expression, then, slowed down the epithelial-to-mesenchymal transition (EMT) progression.

CONCLUSIONS: CASC2 was downregulated in CCA tissues and cells. It could inhibit cell proliferation, invasion, migration and EMT *via* sponging miR-18a/SOCS5 axis. This might provide a novel target for CCA diagnosis and treatment.

Key Words:
CASC2, CCA, Proliferation, Metastasis, EMT, MiR-18a/SOCS5.

Introduction

Cholangiocarcinoma (CCA) originates from biliary epithelial cells, and its incidence in malignant tumors of the hepatobiliary system is second only to hepatocellular carcinoma[1]. In recent years, the incidence of CCA has increased year by year, and the growth rate has surpassed that of hepatocellular carcinoma[2]. Due to its poor prognosis and high mortality, CCA has become one of the major malignant tumors that threaten human health[3]. However, the specific mechanism of CCA tumorigenesis and development remains unclear.

Long non-coding RNA (lncRNA), as a series of non-coding RNAs larger than 200 nt, is characterized by lacking of protein coding function[4]. At present, many lncRNAs have been studied to participate in the occurrence and progression of several tumors. Their abnormal expression in tumor tissue indicates that they have the potential to become the targets for tumor detection and therapy. In addition, lncRNAs have been shown to regulate tumor cell behavior, including cell proliferation, adhesion, apoptosis, metastasis, and drug resistance[5,6]. In fact, lncRNA NNT-AS1 is elevated-expressed in CAA and facilitates tumor progression *via* promoting EMT. The upregulation of lncRNA-ATB and ZFAS1 could accelerate cell growth and metastasis of CCA relatively by modulating miR-200c and miR-296-5p. In tumorigenesis of CCA, lncRNA DANCR epigenetically silenced FBP1 to regulate tumor progression. Also, lncRNA HOTAIR, SPRY4-IT1 and CCAT2 perform tumor promoting function in CCA and forecasted poor prognosis[7-13]. On the contrary, lncRNA MEG3 inhibits CAA cell growth and metastasis through Bmi1/RNF2. Besides, lncRNA MIR22HG negatively modulates Wnt/β-catenin signaling pathway to reduce cell proliferation and migration in CCA[14,15]. LncRNA CASC2 has been identified to inhibit human gliomas, hepatocellular carcinoma, breast cancer, papillary thyroid carcinoma, and adenocarcinoma progression, but its role in CCA has not been studied before[16-22].

Here, CASC2 expression in CCA tissues and cells was detected. Also, influence of CASC2 in cell proliferation was studied using colony forma-

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tion and CCK-8 assays. Effect of CASC2 in cell metastasis was also analyzed. Moreover, miR-18a/SOSC5 was verified as an effective target of CASC2 in CCA. This study first demonstrated the regulatory relationship between CASC2/miR-18a, and indicated its important role in the development of CCA, suggesting that CASC2 has the potential to be a biomarker for CCA, but the mechanism still needed further study.

Patients and Methods

Clinical Tissue Samples

A total of 43 cases of CCA and adjacent normal tissues (>5 cm from the side of CCA) were collected from The First Hospital of Jilin University (29 males and 14 females, with an average age of 56.47 years). This study was approved by the Ethics Committee of The First Hospital of Jilin University. Signed written informed consents were obtained from all participants before the study. Tissues were stored in liquid nitrogen for the next use.

Cell Culture and Transfection

Human intrahepatic biliary epithelial cells (HIBEC) and a series of human CCA cell lines (HUCCT1, RBE, CCLP, QBC939) were obtained from the Clinical Medical Research Center of Nanjing Medical University. All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Rockville, MD, USA) and cultured in a 37°C cell culture incubator containing 5% CO_2 with humid air.

Cell transfection was done using shRNA and lentivirus for CASC2 and miR-18a mimics from GenePharma Co. Ltd (Shanghai, China). HUCCT1 or QBC939 cells were transfected with shRNA, Lentivirus, or mimics, using polybrene (Genepharma, Shanghai, China) or lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). After transfection, the efficiency was observed using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR).

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from the CCA tissues and cells using TRIzol reagent (TaKaRa, Otsu, Shiga, Japan). The extracted RNA was then reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using the PrimeScript™ RT kit (TaKaRa, Otsu, Shiga, Japan). Then, the SYBR® PremixEx Taq™ (TaKaRa, Otsu, Shiga, Japan) was used to quantify the relative levels of CASC2 or miR-18a with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. Primer sequences used for CASC2 and miR-18a: CASC2 forward 5'-TTGGTCTCGGGAACGTGAAGG-3', reverse 5'-TCCCTAGGACCCGCCACTG-3'; miR-18a: forward 5'-AACACGCTAAGGTGCATCTAGTG-3', reverse 5'-CAGTGCAGGGTCCGAGGT-3'; GAPDH: forward 5'-AATGGACAACTGGTCGTGGAC-3', reverse 5'-CCCTCCAGGGGATCTGTTTG-3'. PCR was performed on Prime 7900HT Fast software (ABI PRISM, Applied Biosystems, Foster City, CA, USA) and relative RNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. All the experiments were performed three times.

Cell Counting Kit-8 (CCK-8) Assay

QBC939 or HUCCT1 cells after treatment were seeded and cultured in 96-well plates. At 24, 48, 72, and 96 h after cultured at 37°C in a 5% CO_2 incubator, 10 μL of CCK-8 reagent (Dojindo Molecular Technologies, Kumamoto, Japan) was added to each well, and the cells were cultured for over 2 h in the darkness. Finally, the absorbance value of each well at a wavelength of 470 nm was measured using spectrophotometer. Six replicates were set in each group, and the experiment was repeated for three times.

Colony Formation Assay

QBC939 or HUCCT1 cells after treatment were prepared as single cell suspensions, and seeded in 6 cm culture dishes in the number of 3000 cells per well. After 15 days of culture in DMEM containing 10% FBS, the cells were washed with phosphate-buffered saline (PBS), fixed with paraformaldehyde, and stained with crystal violet. The number of colonies containing more than 50 cells was counted under a microscope. Each set of experiments was repeated three times.

Transwell and Matrigel Assay

The 8-μm chambers (Millipore, Billerica, MA, USA) were purchased for the transwell and Matrigel assays. For transwell assay, QBC939 or HUCCT1 cells after treatment cultured in DMEM medium without FBS were inoculated on the upper layer of the chamber. Then, DMEM medium containing 10% FBS was added to the lower layer of the chamber. After culture for 36 h, the chamber was taken out, fixed in ice methanol, and cleaned the upper layer cells using cotton swabs.

Five visual fields were taken under microscope (100´) and the average number of cells in the fields was counted. For Matrigel assay, the upper layer of the chamber was covered with Matrigel (BD Biosciences, San Jose, CA, USA). Next, the cells were cultured for 48 h after planted and the other steps were the same as the transwell assay.

Dual-Luciferase Assay

Wild-type (WT) or mutant (Mut) CASC2 and SOCS5 sequences that bind to miR-18a were cloned into the pG3 Basic vector (Promega, Madison, WI, USA). A total of 10 μg of pLuc-CASC1-wt-/TFDP2 or pLuc-mut-CASC2/tD2 was co-transfected into 293T cells with miR-18b mimic or negative control (GenePharma, Shanghai, China). After 24 h of culture, the cells were lysed and assayed for relative Luciferase activity.

Western Blot

Total protein of cells was extracted using radioimmunoprecipitation assay (RIPA) reagent (Beyotime, Shanghai, China) containing phenylmethylsulfonyl fluoride (PMSF) protease inhibitor (Beyotime, Shanghai, China). Bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China) was used to measure the protein concentration. Western blot was used to measure the protein expression level. Thereafter, 20 μg of total protein were added to the 10% sodium dodecyl sulphate (SDS) page and separated by electrophoresis. Then, the protein was transferred to the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% no-fat milk, the membranes were immersed in the first antibody overnight at 4°C, and then, in the horseradish peroxidase (HRP)-labeling second antibody after washing with Tris-Buffered Saline (TBS). Finally, the gray value of the membrane was imaged and analyzed using iBright (Invitrogen-Life Technologies, Carlsbad, CA, USA) with an enhanced chemiluminescence (ECL) kit (Bio-Rad lab, Hercules, CA, USA).

Statistical Analysis

The experimental data were expressed as mean ± standard deviation ($\bar{x}$ ± SD). Statistical analysis was performed with Statistical Product and Service Solutions (SPSS) 20.0 software (IBM Corp., Armonk, NY, USA). The differences between the two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered to have a significant difference.

Results

LncRNA CASC2 Expressed Significantly Lower in CCA Tissues and Cells

To verify the expression of CASC2 in CCA tissues, 43 paired CCA tissues and adjacent normal tissue samples were collected. The relative expression of CASC2 was analyzed using qRT-PCR. As clearly shown in the Figure 1A, CASC2 was expressed at a significantly lower level in the CCA group comparing with the normal group. Also, in four CCA cell lines (HUCCT1, RBE, CCLP, QBC939), the expression of CASC2 showed a significant decrease when compared with the human intrahepatic biliary epithelial cells (HIBEC) (Figure 1B). These results indicated that CASC2 might function as a tumor suppressor in CCA.

For further experiments for investigating functions of CASC2 in CCA, CASC2 level was overexpressed in QBC939 cells and CASC2 expression was inhibited in HUCCT1 cells with using LV-CASC2 and shRNA-CASC2 relatively. By comparing with each control group, QBC939 cells expressed markedly higher CASC2 level after LV-CASC2 treatment while HUCCT1 cells had lower CASC2 expression after shRNA-CASC2 transfection (Figure 1C, 1D).

Abnormal CASC2 Expression Affected Cell Proliferation of CCA Cells

Next, to further study the influence of CASC2 on cell proliferation, colony formation and CCK-8 assays were employed. By comparing to the control group, QBC939 cells formed fewer colonies after LV-CASC2 treatment (Figure 2A, 2B). On the contrary, HUCCT1 cells showed increased colony formation ability after CASC2 inhibition (Figure 2C, 2D). Similarly, CCK-8 assay showed that QBC939 cells with overexpressed CASC2 grew slower than the control group, while HUCCT1 cells with downregulated CASC2 exhibited enhanced proliferation ability (Figure 2E, 2F). These results suggest CASC2 can inhibit cell proliferation of CCA cells.

CASC2 Influenced Cell Invasion and Migration of CCA Cells

The effects of CASC2 on cell invasion and migration were elucidated by transwell and Matrigel assays. Clearly, upregulation of CASC2 reduced invaded QBC939 cell number but the inhibition of CASC2 enhanced HUCCT1 cell invasion when comparing to the control group (Figure 3A, 3B, 3C, 3D). Analogously, by comparing to each

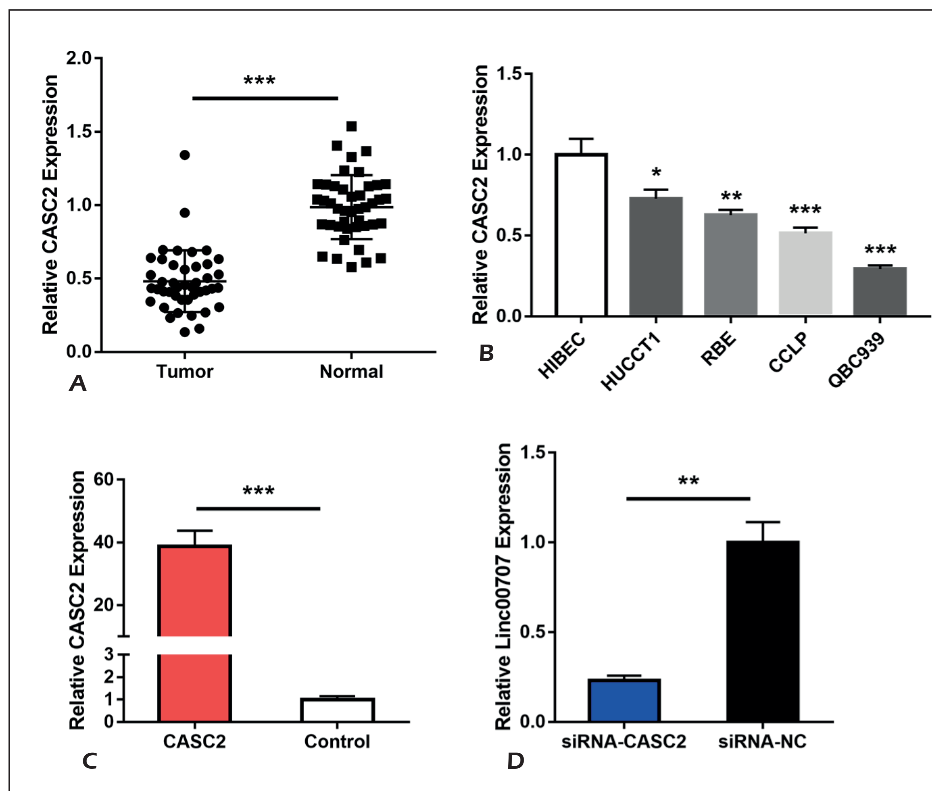

Figure 1. LncRNA CASC2 is low-expressed in CCA tissues and cells. **c**, QRT-PCR shows the lncRNA CASC2 expression level in total of 43 CCA tissues and paired normal tissues. **B**, CASC2 expression level in CCA cell lines (QBC939, RBE, CCLP, HUCCT1) and human intrahepatic biliary epithelial cells (HIBEC). **C**, LV-CASC2 (CASC2) and LV-control (Control) are transfected into QBC939 cells. **D**, Oligonucleotides targeting CASC2 (siRNA-CASC2) and negative controls (siRNA-NC) are transfected into HUCCT1 cells. ***p<0.001, **p<0.01, *p<0.05 compared to control group.

control group, cell migration ability of QBC939 cells was significantly decreased by LV-CASC2 treatment when HUCCT1 improved by shRNA-CASC2 transfection (Figure 3A, 3B, 3C, 3D). These experiments indicate that CASC2 can inhibit cell invasion and migration of CCA cells.

MiR-18a Was a Potential Target for CASC2 in CCA

CASC2 was demonstrated to inhibit cell proliferation and metastasis of CCA cells, so the underlying mechanism of CASC2 in CCA was further explored. Several studies have identified that lncRNA could function as ceRNA *via* sponging targeted miRNA to influence the downstream protein expression. According to this, some databases, including StarBase, Diana, and RBPDB, were searched, and it was found that miR-18a was a potential target for CASC2 (Figure 4A). To verify this assumption, Dual-Luciferase activity assay was conducted using sequence containing wild type or mutant miR-18a binding region of CASC2. The wild type group showed evident reduced Luciferase activity, while no difference in the mutant group after miR-18a mimics treatment was observed (Figure 4B). Moreover, using qRT-PCR, the miR-18a expression in established QBC939 and HUCCT1 cells was detected. Overexpression of CASC2 markedly decreased the miR-18a expression in QBC939 cells compared to the LV-control group (Figure 4C). However, downregulation of CASC2 showed increased miR-18a than the control group (Figure 4D). These suggest that miR-18a may be a target gene for CASC2 in CCA.

CASC2 Inhibited EMT Via MiR-18a/SOCS5 Axis

MiR-18a was verified as a target for CASC2. Furthermore, the downstream molecular for CASC2/miR-18a was detected. Using several databases (miRDB, Diana, TargerScan, miRWalk), SOCS5 was chosen as a direct target for miR-18a. Also, the 3'-UTR of SOCS5 containing the miR-

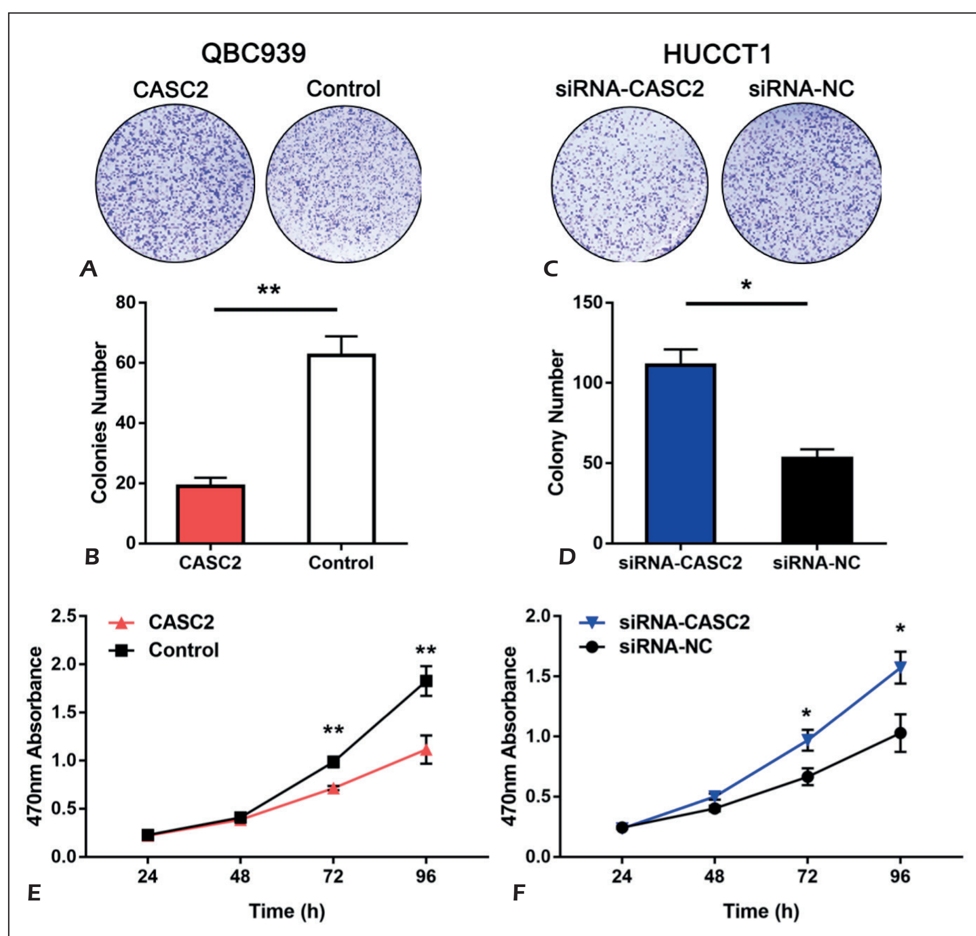

Figure 2. CASC2 affects the proliferation of CCA cells in vitro. **A-D**, Clone formation assay shows the proliferation ability of QBC939 cells transfected with LV-CASC2 and LV-control or HUCCT1 cells transfected with siRNA-CASC2 and siRNA-NC. (magnification: 40×) **E-F**, CCK-8 assays show the proliferation ability of QBC939 cells transfected with LV-CASC2 and LV-control or HUCCT1 cells transfected with siRNA-CASC2 and siRNA-NC. ***p*<0.01, **p*<0.05 compared to control group.

18a binding site was mutated and plasmid of wild type and mutant group was constructed for the Luciferase assay (Figure 5A). Compared with that in the mutant group which displayed no difference between the miR-18a overexpression group and the negative control group, Luciferase activity in the wild-type group decreased significantly in the miR-18a mimics treated group (Figure 5B). These results indicated that SOCS5 might act as a target for miR-18a. Then, the protein expression of SOCS5 was measured using Western blot in QBC939 and HUCCT1 cells. It was found that overexpression of CASC2 increased the expression of SOCS5 protein in QBC939 cells while inhibition of CASC2 reduced the SOCS5 level in HUCCT1 cells (Figure 5C, 5D, 5E). Next, according to several studies that SOCS5 could affect the EMT of cancer cell, the epithelial marker E-cadherin and mesenchymal marker N-cadherin, and Vimentin expressions in experimental cells were examined[23]. The results showed that the increase of CASC2 level markedly improved the expression of E-cadherin but reduced the level of N-cadherin and Vimentin compared to the control group (Figure 5C, 5D). By contrast, the decrease of CASC2 level in HUCCT1 cells showed completely reverse function (Figure 5C, 5E). All the results suggest that CASC2 can sponge miR-18a to promote the expression of SOCS5 and then inhibit the progression of EMT.

Discussion

CCA is characterized by insidious onset, lack of ideal early diagnostic markers, insensitivity to conventional radiotherapy and chemotherapy, and low surgical resection rate. The incidence of CCA

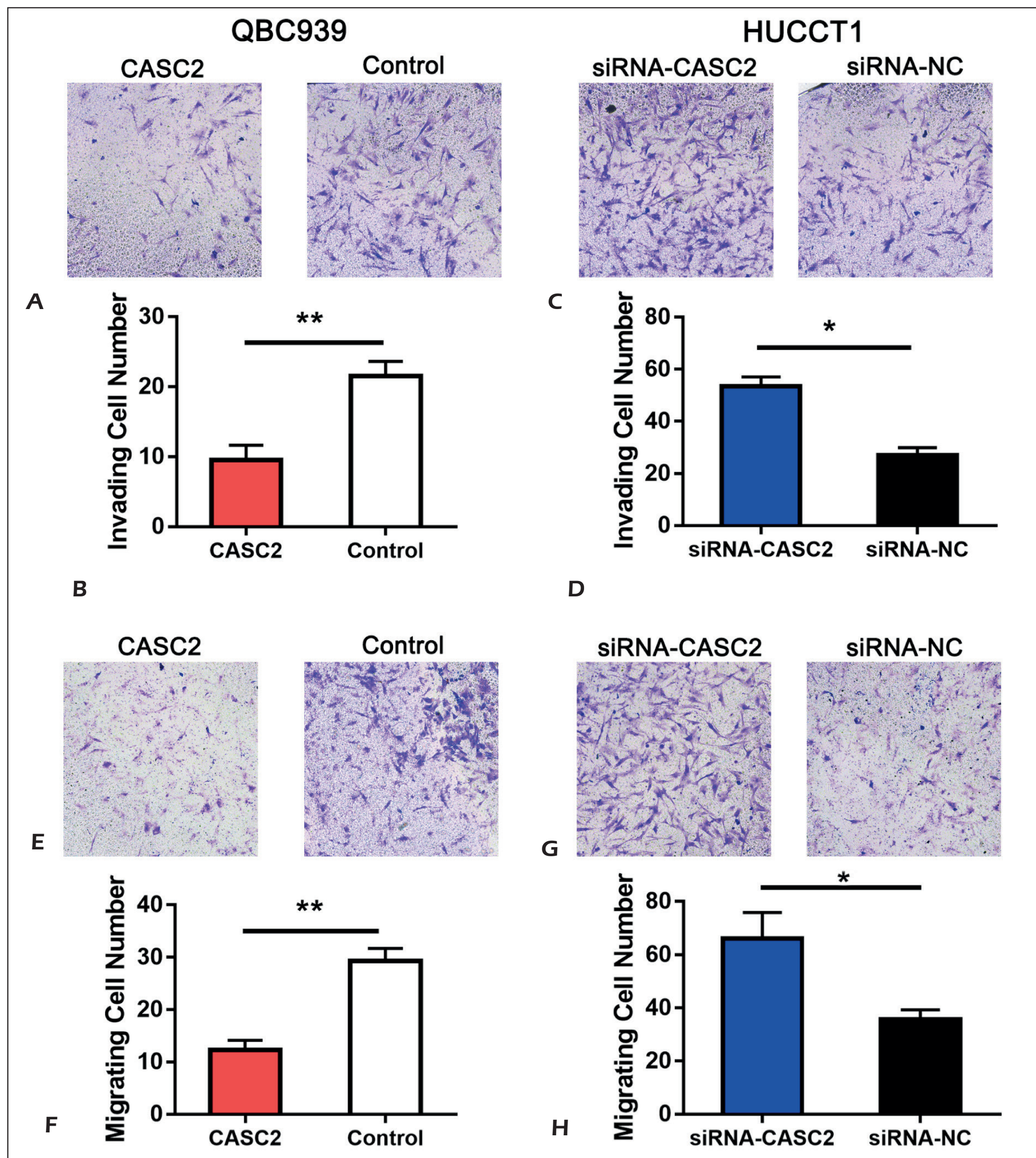

Figure 3. CASC2 influences the invasion and migration of CCA cells. **A-B**, Transwell invasion assay indicates the invaded cell number in QBC939 cells (magnification: 40×). **C-D**, Transwell invasion assay indicates the invaded cell number in HUCCT1 cells (magnification: 40×). **E-F**, Matrigel assay shows the migrated cell number in QBC939 cells (magnification: 40×). G, H, Matrigel assay shows the migrated cell number in HUCCT1 cells (magnification: 40×). $^{**}p<0.01$, $^{*}p<0.05$ compared to the control group.

worldwide increased at a rate of 5% per year[24,25], and its prognosis remains extremely poor, with a 5-year survival rate of less than 5%. Actively exploring the mechanism of CCA development and seeking new clinical diagnosis and treatment methods have become concern and urgent problems[26]. LncRNAs have been identified abnormally expressed in many cancers and the function of lncRNA in cancer has been hotspots of cancer research[6,27]. It has been verified that the regulation of lncRNAs plays important roles in the occurrence and development of CCA. Therefore, it is suggested that lncRNAs have the potential to become biomarkers for CCA diagnosis, treatment, and prognosis prediction. LncRNA CASC2 is located at chromosome 10q26.11, which has

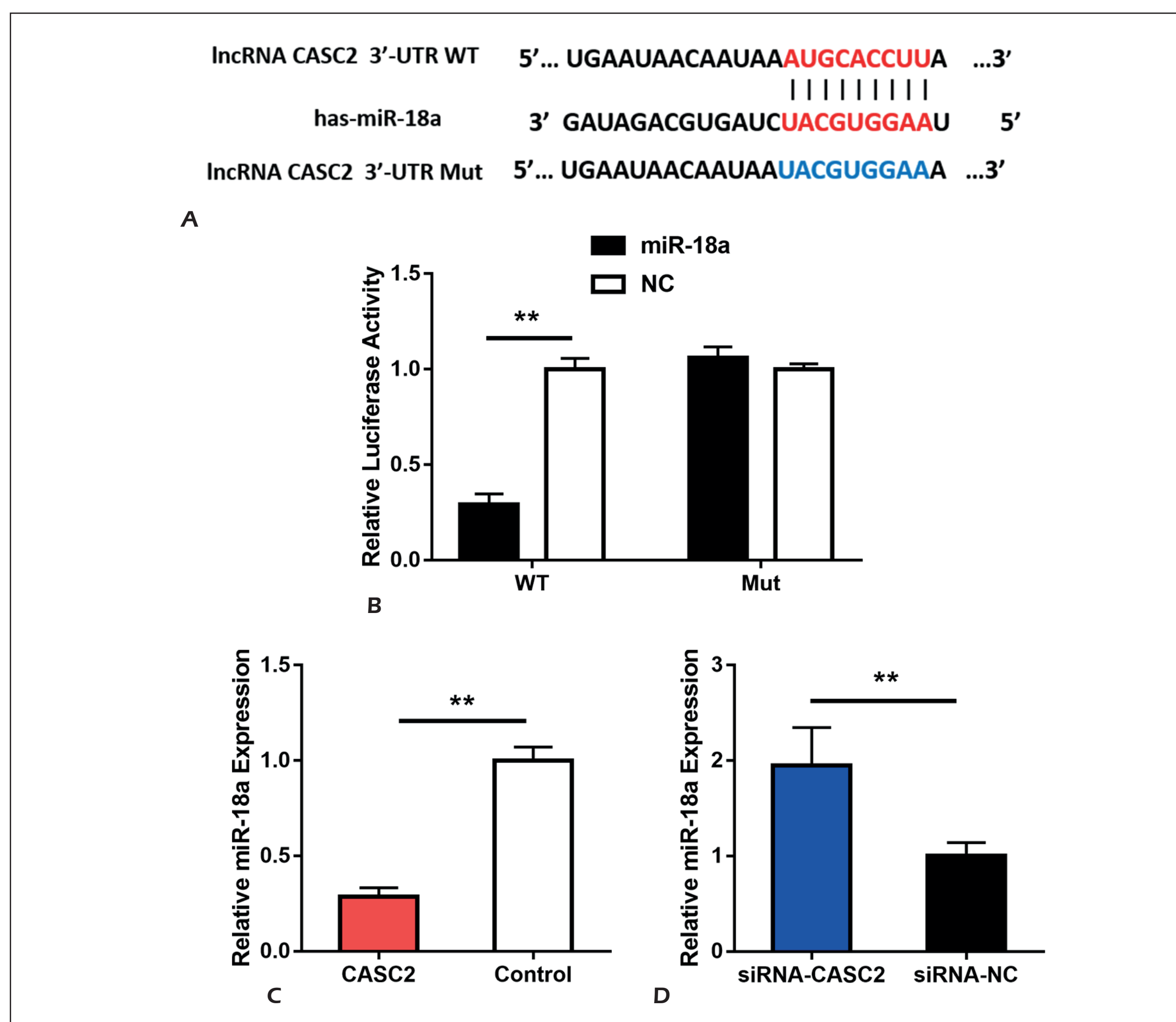

Figure 4. CASC2 functions as a sponge of miR-18a. **A**, The sequences of CASC2 binding with miR-18a, including wild type and mutant group. **B**, Luciferase reporter assay verifies the molecular bound within CASC2 and miR-18a. **C-D**, QRT-PCR shows the miR-18a expression level in experimental QBC939 or HUCCT1 cells. $^{**}p<0.01$ compared to control group.

been identified lowly expressed in many kinds of tumors, such as hepatocellular cancer, breast cancer, lung adenocarcinoma, etc[16,17,21,28]. However, the specific expression and function of CASC2 in CCA still remain unclear. In this study, CASC2 was found to have a cancer suppressing effect in CCA, suggesting that CASC2 may provide a new target for CCA treatment.

In order to clarify the specific function and molecular mechanism of CASC2 in CCA, CASC2 in CCA cells was upregulated or knocked out. Functional experiments showed that the proliferation, invasion, and migration of QBC939 cells were significantly inhibited by CASC2 overexpression. However, cell growth and metastasis of HUCCT1 cells were significantly promoted by CASC2 inhibition. These results indicated that CASC2, as in many other tumors, played a role in inhibiting tumor cell progression. Considering that lncRNA could act as competitive endogenous RNA (ceRNA), we then regulated the downstream gene expression and affected the corresponding biological function by competitively binding targeted miRNA. Therefore, according to the bioinformatics analysis, it was predicted that there was a complementary region between CASC2 and miR-18a. In other words, CASC2 might play a role by downregulating miR-18a. The results of Dual-Luciferase showed that there was a direct binding relationship between CASC2 and miR-18a. After

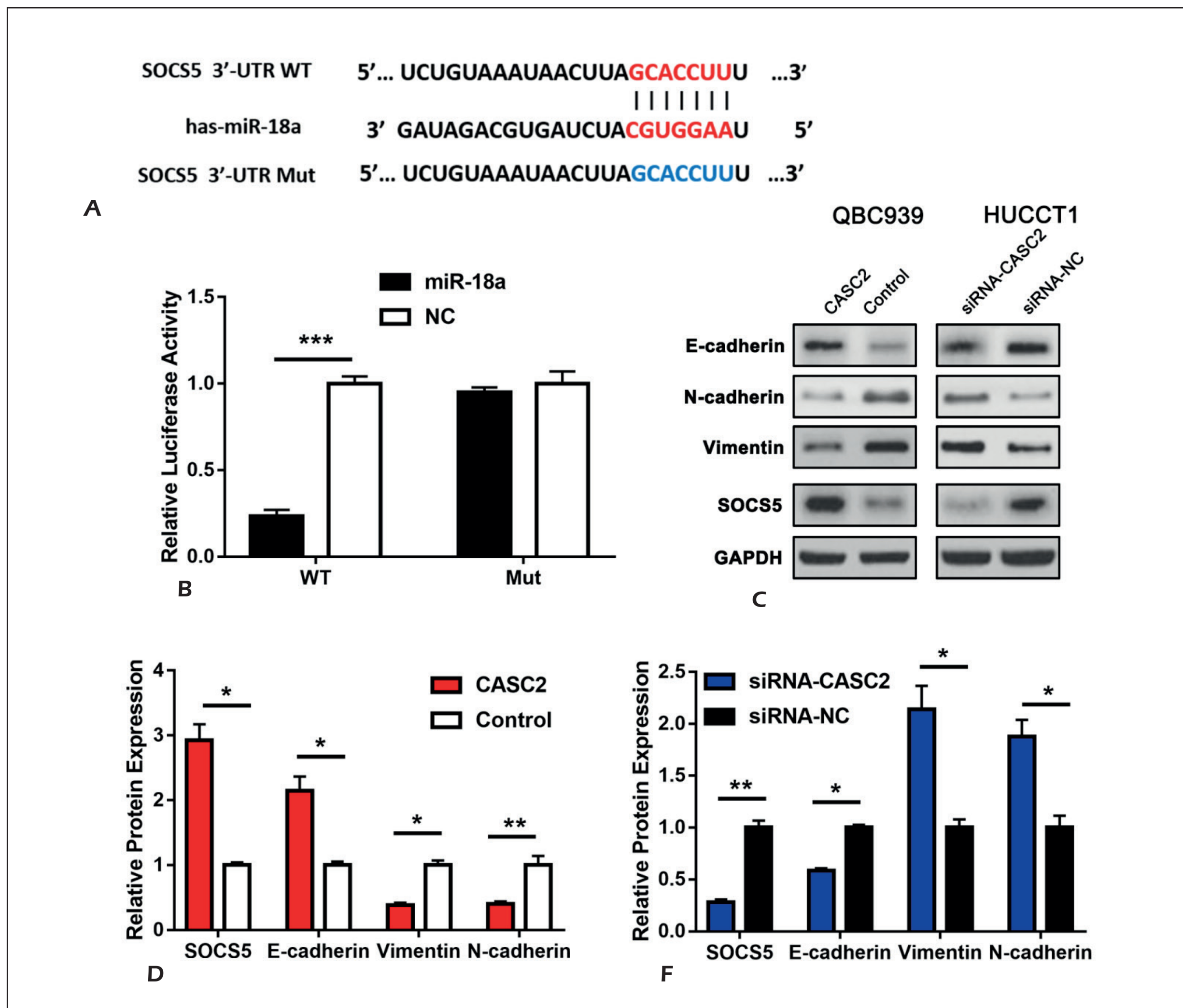

Figure 5. CASC2 inhibits EMT via miR-18a/SOCS5 axis. **A**, The sequences of SOCS5 mRNA 3'-UTR and miR-18a, including wild type and mutant binding site. **B**, Luciferase reporter assay indicates the molecular bound within SOCS5 and miR-18a. **C-F**, Western blot assay indicates the SOCS5 and EMT markers protein expression in established QBC939 cells and HUCCT1 cells. ***p<0.001, **p<0.01 *p<0.05 compared to control group.

knocking out CASC2, the expression of miR-18a increased, but after overexpressing CASC2, miR-18a level decreased. MiR-18a functions as oncogene in many cancers, including nasopharyngeal carcinoma, prostate cancer, and colorectal cancer[29-31]. Suppressor of cytokine signaling (SOCS) family is a class of protein molecules that have negative feedback regulation on cytokine signaling pathways and participate in the signal regulation of a variety of cytokines, growth factors and hormones[32]. Next, it was observed that SOCS5 was a direct target for miR-18a in CCA. More importantly, in CASC2 overexpressed QBC939 cells, SOCS5 protein level increased while in CASC2 inhibited HUCCT1 cells, SOCS5 expression reduced. These demonstrated that SOCS5 was a target for CASC2/miR-18a in CCA.

EMT is a process in which epithelial cells transformed into mesenchymal cells combined with associated genetic and phenotypic changes, which is often activated during tumor invasion and metastasis. EMT plays an important role in the occurrence and development of CCA, especially early metastasis[33,34]. SOCS5 has previously been reported to suppress EMT process in several cancers[23]. Therefore, the expression of EMT-associated protein markers was also tested and it was found that the overexpression of CASC2 significantly inhibited the EMT process of CCA, and after the CASC2 was knocked down, the EMT pro-

cess of CCA was significantly increased. These show that the CASC2/miR-18a/SOCS5 regulatory axis could inhibit the EMT process of CCA cells and thus the tumor metastasis process.

Conclusions

Taken together, this study revealed for the first time that the expression of lncRNA CASC2 was downregulated in CCA, and CASC2 could inhibit CCA cell proliferation, invasion, migration and EMT *via* competitively sponging miR-18a to promote the expression of SOCS5. These might provide a novel target for the future diagnosis, treatment, and prognosis prediction for CCA.

Acknowledgements

This study was supported by National Natural Science Foundation of China (Grant No. 81700205).

Conflict of Interests

The authors declare that they have no conflict of interests.

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