MicroRNA-101 inhibits the migration and invasion of intrahepatic cholangiocarcinoma cells via direct suppression of vascular endothelial growth factor-C

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Abstract. MicroRNAs (miRs) have important roles in the pathogenesis of human malignancy. It has previously been suggested that deregulation of miR-101 is associated with the progression of intrahepatic cholangiocarcinoma (ICC); however, the exact role of miR-101 in the regulation of ICC metastasis remains largely unknown. The present study demonstrated that the expression levels of miR-101 were significantly decreased in ICC tissue, as compared with matched adjacent normal tissue. Furthermore, miR-101 was downregulated in the ICC-9810 human ICC cell line, as compared with in the normal human intrahepatic biliary epithelial cell (HIBEC) line. Vascular endothelial growth factor (VEGF)-C was identified as a target gene of miR-101 in ICC-9810 cells. The expression of VEGF-C was negatively regulated by miR-101 at the post-transcriptional level in ICC-9810 cells. Further investigation demonstrated that overexpression of miR-101 markedly suppressed the migration and invasion of ICC-9810 cells, and these effects were similar to those observed following VEGF-C knockdown. Conversely, restoration of VEGF-C reversed the inhibitory effects of miR-101 overexpression on ICC-9810 cell migration and invasion, thus suggesting that miR-101 may suppress ICC-9810 cell migration and invasion, at least partly via inhibition of VEGF-C. It was also demonstrated that the mRNA and protein expression levels of VEGF-C were frequently upregulated in ICC tissue and cells, and its expression level was inversely correlated with that of miR-101 in ICC tissue. In conclusion, the present study identified important roles for miR-101 and VEGF-C in ICC, suggesting that miR-101/VEGF-C signaling may be a promising diagnostic and/or therapeutic target for ICC.

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Introduction

Intrahepatic cholangiocarcinoma (ICC) is a rare type of primary liver cancer that originates from cholangiocytes, and is ranked as one of the top five causes of cancer-associated mortality. Furthermore, the 5-year survival rate of patients with ICC is poor, predominantly due to high recurrence and metastasis (1,2); therefore, further investigation regarding potential diagnostic and therapeutic targets may aid in reducing the mortality rate of ICC.

Vascular endothelial growth factor (VEGF)-C is an important member of the VEGF family, which has been reported to be associated with the progression of ICC (3). High expression of VEGF-C is significantly correlated with vascular invasion, lymph node metastasis, and the presence of positive surgical margins, as well as poor survival rate (4). However, the regulatory mechanisms of VEGF-C in ICC remain to be elucidated.

MicroRNAs (miRs) are a class of non-coding RNAs, 18-25 nucleotides in length. It has been well established that miRs directly bind to the 3'-untranslated region (UTR) of their target mRNAs, leading to mRNA degradation or inhibition of protein translation (5). By regulating the expression of their target genes, miRs are involved in the regulation of cell survival, proliferation, differentiation and migration (6). Furthermore, miRs have important roles in the development and progression of various types of human cancer, including ICC (7,8). A previous study demonstrated that low expression of miR-124, regulated by the hepatitis C virus core protein, was able to enhance the migration and invasion of ICC cells (9). In addition, miR-200c has been shown to activate epithelial-mesenchymal transition via direct inhibition of neural cell adhesion molecule 1 in ICC (10).

Reduced expression of miR-101 has been detected in numerous types of human cancer, and is associated with the invasion and progression of malignancies (11). Zhang *et al* (12) demonstrated that miR-101 was markedly downregulated in cholangiocarcinoma specimens and cell lines, as compared with in non-cancerous biliary epithelial cells, suggesting that downregulation of miR-101 may be involved in the

development of cholangiocarcinoma. However, whether miR-101 participates in the progression of ICC, as well as the underlying molecular mechanism, remain to be elucidated.

The present study aimed to explore the molecular mechanisms by which miR-101 and VEGF-C mediate the migration and invasion of ICC cells.

Materials and methods

Tissue specimen collection. The present study was approved by the Ethical Committee of The Third Xiangya Hospital of Central South University (Changsha, China). A total of 15 ICC tissue samples and matched adjacent normal tissue samples were obtained from the Department of Hepatobiliary and Pancreatic Surgery, the Third Xiangya Hospital of Central South University. The patients providing the samples consisted of 11 males and 4 females with an average age 53.5 years old (41-64 years old). ICC was diagnosed by doctors from the Department of Pathology and tissues were obtained by surgical resection. The patients were recruited between June 2011 and January 2012. All patients provided their written consent for the present study. All tissues were immediately snap-frozen in liquid nitrogen following surgical removal, and stored at -70˚C until further use.

Cell culture. The ICC-9810 human ICC cell line and normal human intrahepatic biliary epithelial cells (HIBEC) were obtained from the Institute of Cell Biology at the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen Life Technologies) at 37˚C in a humidified incubator containing 5% CO_2.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). For mRNA detection, total RNA was extracted from cells and tissues using TRIzol® reagent (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. The tissues were homogenized with liquid nitrogen in a mortar (Ziyi Company, Shanghai, China). RevertAid First-Strand cDNA Synthesis kit (Fermentas, Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to reverse transcribe RNA into cDNA (1 μg used). Subsequently, iQ™ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to perform qPCR with the ABI 7500 thermocycler (Applied Biosystems Life Technologies, Foster City, CA, USA). The cycling conditions were as follows: 95˚C for 5 min, followed by 40 cycles of 95˚C for 30 sec and 60˚C for 30 sec. The sequences of the specific primers from Shanghai Shenggong Co., Ltd. (Shanghai, China) were as follows: VEGF-C, forward 5'-GGCTGGCAACATAACAGAGAA-3', reverse 5'-CCCCACATCTATACACACCTCC-3'; and GAPDH, forward 5'-ACAACTTTGGTATCGTGGAAGG-3', and reverse 5'-GCCATCACGCCACAGTTTC-3'. GAPDH was used as an internal reference. Relative expression was analyzed using the $2^{-\Delta\Delta Ct}$ method.

For miR detection, total RNA was extracted using TRIzol® reagent, according to the manufacturer's instructions. miRNA Reverse Transcription kit (Life Technologies) was used to convert RNA into cDNA (1 μg used). Subsequently, qPCR was conducted using the miRNA QRT-PCR Detection kit (GeneCopoeia, Rockville, MD, USA) and the ABI 7500 thermocycler. The cycling conditions were as follows: 95˚C for 5 min, followed by 40 cycles of 95˚C for 30 sec and 60˚C for 30 sec. U6 was used as an internal reference. Relative expression was analyzed using the $2^{-\Delta\Delta Ct}$ method.

Western blotting. Tissues and cells were solubilized in cold radioimmunoprecipitation lysis buffer (Invitrogen Life Technologies). The tissues were homogenized with liquid nitrogen in a mortar (Ziyi Company, Shanghai, China). Proteins were separated by 10% SDS-PAGE (Beyotime Institute of Biotechnology, Shanghai, China), and transferred onto a polyvinylidene difluoride (PVDF; Invitrogen Life Technologies) membrane. The PVDF membrane was then incubated with phosphate-buffered saline containing 5% milk at 4˚C overnight. Subsequently, the PVDF membrane was incubated with monoclonal mouse anti-human VEGF-C (ab63221; 1:100) and monoclonal mouse anti-human GAPDH (ab8245; 1:100) primary antibodies (Abcam, Cambridge, UK) at room temperature for 3 h. The membrane was then incubated with rabbit anti-mouse secondary IgG antibodies (Abcam) at room temperature for 1 h. An Enhanced Chemiluminescence kit (Pierce Biotechnology, Inc., Rockford, IL, USA) was then used to perform chemiluminescent detection. The relative protein expression was analyzed using Image-Pro plus software 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) and was presented as the density ratio versus GAPDH.

Transfection. Lipofectamine® 2000 (Life Technologies) was used to perform cell transfection, according to the manufacturer's instructions. For miR-101 and VEGF-C functional analysis, ICC-9810 cells ($5x10^6$ cells) were transfected for 48 h at 37˚C with 100 mM each of scrambled miR as a negative control (NC), miR-101 mimics, miR-101 inhibitor (Life Technologies), VEGF-C-small interfering (si)RNA, or VEGF-C plasmid (Nlunbio, Changsha, China), respectively. Non-transfected ICC-9810 cells were used as control.

Bioinformatics analysis. Bioinformatics analysis was conducted to predict the putative targets of miR-101 using Targetscan online software (www.targetscan.org).

Dual luciferase reporter assay. A mutant (MUT) 3'-UTR of VEGF-C was generated using the Directed Mutagenesis kit (Stratagene, Agilent Technologies, Inc., Santa Clara, CA, USA), according to the manufacturer's instructions. The wild type (WT) or MUT 3'-UTR of VEGF-C (Nlunbio) was inserted into the psiCHECK™2 vector (Promega Corporation, Madison, WI, USA). Subsequently, ICC-9810 cells ($5x10^6$ cells) were transfected with psiCHECK™2-WT VEGF-C 3'-UTR or psiCHECK™2-MUT VEGF-C 3'-UTR vector, with or without miR-101 mimics (100 nM), and then incubated at 37˚C in an atmosphere containing 5% CO_2 for 48 h. The Dual-Luciferase Reporter Assay system (Promega Corporation) was then used to determine the luciferase activities on an LD400 luminometer (Beckman Coulter, Inc., Brea, CA, USA). *Renilla* luciferase activity was normalized to firefly luciferase activity.

Cell migration and invasion assays. Cell migration and invasion assays were performed using Transwell chambers (BD Biosciences, Franklin Lakes, NJ, USA). For the invasion assay, the Transwell chambers were pre-coated with Matrigel (BD Biosciences), whereas the chambers used for the migration assay were not. A cell suspension containing $5x10^5$ cells/ml was prepared in serum-free media, and 300 μl cell suspension was added into the upper chamber. A total of 500 μl DMEM supplemented with 10% FBS was added to the lower chamber. The cells were incubated for 24 h at 37˚C. A cotton-tipped swab was then used to carefully wipe away the cells that did not migrate or invade through the pores. The filters were fixed in 90% alcohol and stained with crystal violet (Sigma-Aldrich, St. Louis, MO, USA). Cell number was determined in five fields randomly selected under an inverted microscope (IX73-A21PH; Olympus Corporation, Tokyo, Japan).

Statistical analysis. SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used to perform statistical analysis. All data are expressed as the mean ± standard deviation. Differences were analyzed using one-way analysis of variance. $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-101 is downregulated in ICC tissue samples and cell lines. To determine the role of miR-101 in ICC, RT-qPCR was conducted to examine the expression levels of miR-101 in ICC tissue samples. miR-101 was markedly downregulated in the ICC tissue, as compared with in the matched adjacent normal tissue (Fig. 1A). Furthermore, the miR-101 expression levels were determined in ICC cells; miR-101 was also downregulated in the ICC-9810 human ICC cell line, as compared with in the normal HIBECs (Fig. 1B). These findings suggest that deregulation of miR-101 may be associated with the development of ICC.

VEGF-C is a target gene of miR-101. Bioinformatics analysis suggested that VEGF-C is a potential target gene of miR-101. Therefore, the present study investigated whether miR-101 could mediate the expression of VEGF-C in ICC-9810 cells. As presented in Fig. 2A, the miR-101 levels in ICC-9810 cells transfected with miR-101 mimics were significantly upregulated compared with the control group. By contrast, the miR-101 levels in ICC-9810 cells transfected with the miR-101 inhibitor were markedly reduced compared with the control group. In addition, transfection with NC miR did not affect the miR-101 in ICC-9810 cells. These above data indicate that the transfection efficiency was satisfactory. Subsequently, the protein expression levels of VEGF-C were determined using western blotting. As shown in Fig. 2B, upregulation of miR-101 markedly decreased the protein expression levels of VEGF-C, whereas downregulation of miR-101 significantly increased the protein expression levels of VEGF-C in the ICC-9810 cells. To further verify whether miR-101 may directly bind to seed sequences in the VEGF-C 3'-UTR in ICC-9810 cells, WT and MUT VEGF-C 3'-UTRs were generated (Fig. 2C) and a luciferase reporter assay was performed. Luciferase activity was significantly reduced in the cells co-transfected with the WT VEGF-C 3'UTR and miR-101 mimics; however, there was no difference in luciferase activity in the cells co-transfected with the MUT VEGF-C 3'UTR and miR-101 mimics, as compared with the control group (Fig. 2D). These results indicate that VEGF-C is a direct target of miR-101, and the protein expression levels of VEGF-C are negatively regulated by miR-101 in ICC-9810 cells.

miR-101 suppresses ICC cell migration by targeting VEGF-C. Since VEGF-C has been suggested to be involved in ICC metastasis, the present study further examined the roles of miR-101 and VEGF-C in the regulation of ICC cell migration. ICC-9810 cells were transfected with miR-101 mimics, VEGF-C siRNA, or co-transfected with miR-101 mimics and VEGF-C plasmid. As shown in Fig. 3A, transfection efficiency was satisfactory. Upregulation of miR-101 or knockdown of VEGF-C markedly suppressed the migration of ICC-9810 cells; however, the suppressive effects of miR-101 upregulation on ICC-9810 cell migration were significantly attenuated following overexpression of VEGF-C (Fig. 3B). These results suggest that miR-101 may inhibit ICC-9810 cell migration by directly targeting VEGF-C.

miR-101 suppresses ICC cell invasion by targeting VEGF-C. The present study also examined the effects of miR-101 and VEGF-C on ICC cell invasion. Concordant with the cell migration results, upregulation of miR-101 or knockdown of VEGF-C significantly inhibited ICC-9810 cell invasion; however, the suppressive effects of miR-101 overexpression on ICC-9810 cell invasion were reversed following upregulation of VEGF-C (Fig. 4). These results indicate that miR-101 may inhibit ICC-9810 cell invasion via direct inhibition of VEGF-C.

VEGF-C expression is markedly upregulated in ICC tissue samples and cells. The present study further detected the mRNA and protein expression levels of VEGF-C in ICC tissue samples and cell lines. As shown in Fig. 5A and B, the mRNA and protein expression levels of VEGF-C were frequently upregulated in ICC tissue, as compared with in the matched adjacent normal tissue. Furthermore, the mRNA and protein expression levels of VEGF-C were upregulated in the ICC-9810 cells, as compared with in the normal HIBECs (Fig. 5C and D). In addition, an inverse correlation was detected between miR-101 and VEGF-C expression in the ICC tissue (Fig. 5E).

Discussion

miR-101 has previously been demonstrated to act as a tumor suppressor in numerous types of malignant tumors (11,13). The results of the present study demonstrated that miR-101 was significantly downregulated in ICC tissue samples and cell lines. In addition, a novel target gene of miR-101 was identified, VEGF-C, the expression of which was negatively regulated by miR-101 in ICC-9810 cells. These results suggested that miR-101 was able to inhibit the migration and invasion of ICC-9810 cells, at least in part, via direct suppres-

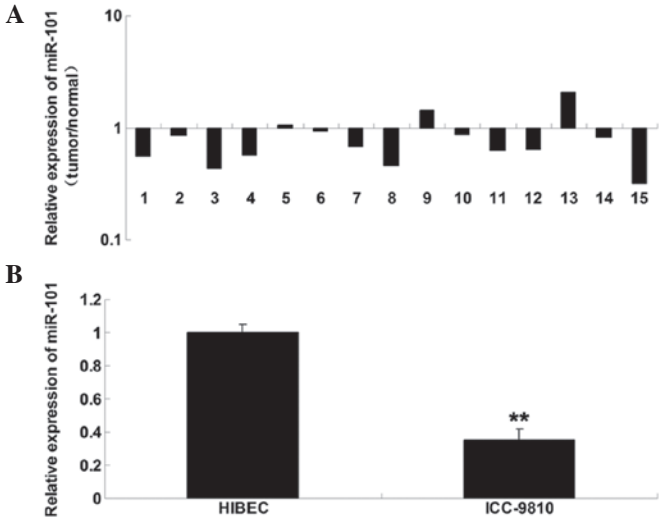

Figure 1. (A) Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to determine the relative expression levels of microRNA (miR)-101 in intrahepatic cholangiocarcinoma (ICC) tumor tissue and matched normal adjacent tissue. (B) RT-qPCR was used to determine the relative expression levels of miR-101 in the ICC-9810 human ICC cell line and the normal intrahepatic biliary epithelial cells (HIBEC). **P<0.01 vs. HIBECs

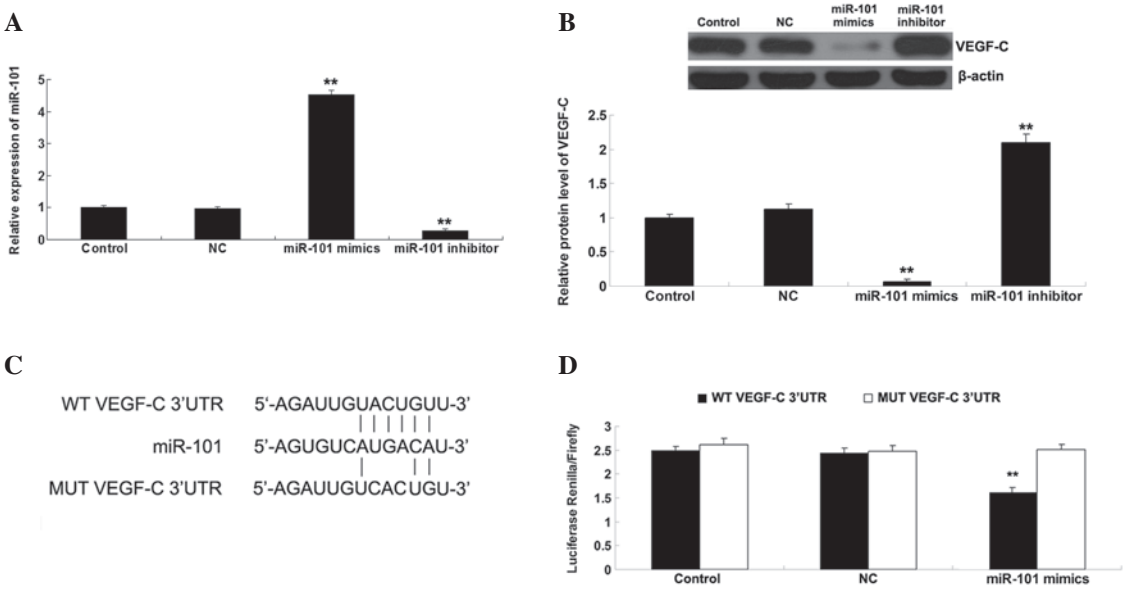

Figure 2. (A) Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to determine the relative expression levels of microRNA (miR)-101 in ICC-9810 intrahepatic cholangiocarcinoma cells transfected with scramble miR as a negative control (NC), miR-101 mimics, or a miR-101 inhibitor. Control, untransfected ICC-9810 cells. (B) Western blot analysis was used to determine the protein expression levels of vascular endothelial growth factor (VEGF)-C in ICC-9810 cells transfected with NC scramble miR, miR-101 mimics or a miR-101 inhibitor. GAPDH was used as an internal reference. Control, untransfected ICC-9810 cells. (C) Seed sequences of miR-101 in the wild (WT) or mutant (MUT) 3'-untranslated region (UTR) of VEGF-C are indicated. (D) Luciferase reporter assay demonstrated that co-transfection of ICC-9810 cells with miR-101 and WT VEGF-C 3'-UTR resulted in a significant decrease in luciferase activity, whereas co-transfection with MUT VEGF-C 3'-UTR and miR-101 mimics resulted in no difference in luciferase activity, as compared with the control group. Control, cells co-transfected with a blank vector and WT VEGF-C 3'-UTR or MUT VEGF-C 3'-UTR. **P<0.01 vs. the control cells.

sion of VEGF-C protein expression. In addition, VEGF-C was shown to be upregulated in ICC tissue samples and cells, and its expression was inversely correlated with miR-101 expression in ICC tissue.

Increasing evidence has demonstrated that miR-101 acts as a tumor suppressor in numerous types of human malignancies (11). The expression of miR-101 has previously been shown to be frequently reduced in various types of cancer, including non-small cell lung cancer cells, gastric cancer, ovarian cancer, endometrial cancer and cervical cancer (14-19). Yan *et al* (20) demonstrated that miR-101 was able to inhibit lung tumorigenesis via suppression of DNA methlytransferase 3a-dependent DNA methylation. Wang *et al* (21) reported that miR-101 may promote the apoptosis of breast cancer cells by targeting Janus kinase 2. In addition, Zhang *et al* (12) demonstrated that miR-101 was notably downregulated in cholangiocarcinoma tissue samples and cell lines, thus suggesting that miR-101 may participate in cholangiocarcinoma progression. However, the detailed role of miR-101 in ICC has yet to be investigated.

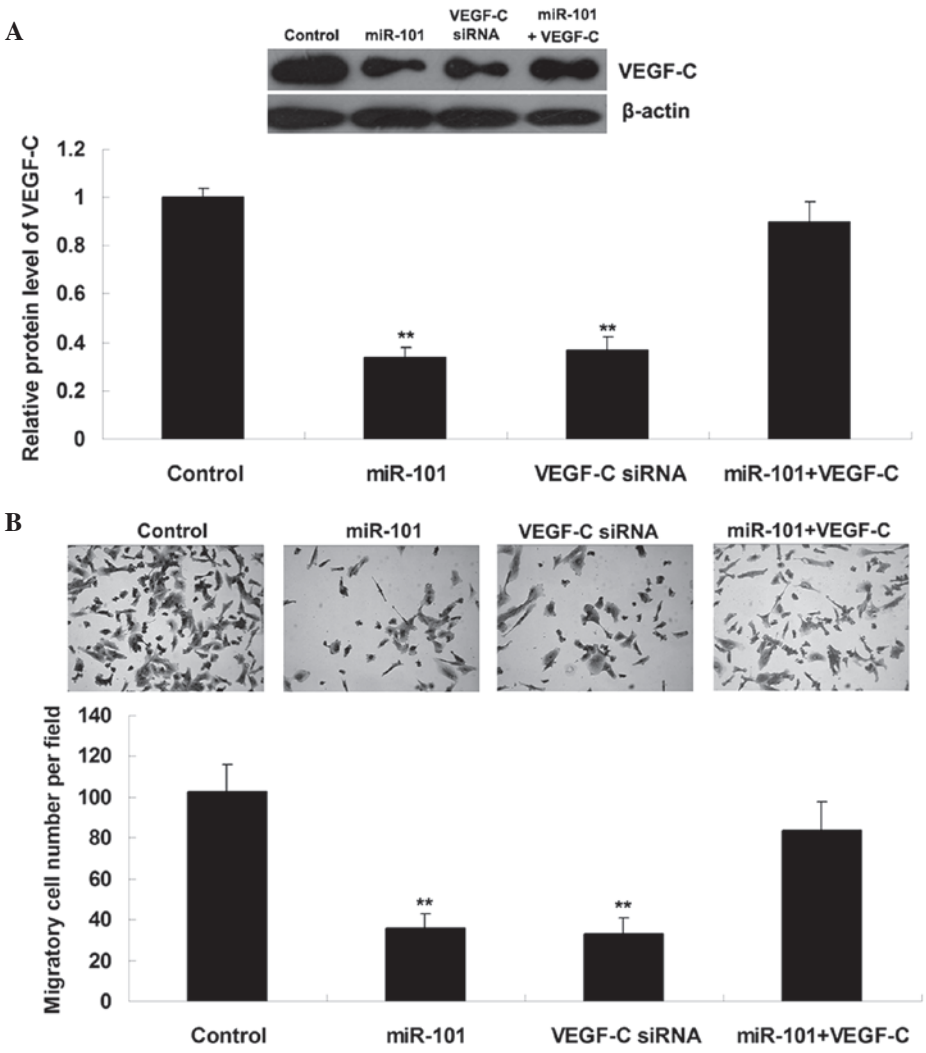

Figure 3. (A) Western blotting was performed to examine the protein expression levels of vascular endothelial growth factor (VEGF)-C in ICC-9810 intrahepatic cholangiocarcinoma cells transfected with microRNA (miR)-101 mimics, VEGF-C small interfering (si)RNA, or co-transfected with miR-101 mimics and VEGF-C plasmid. GAPDH was used as an internal reference. Control, untransfected ICC-9810 cells . **P<0.01 vs. the control cells. (B) Transwell assay was used to determine the migratory capacity of ICC-9810 cells transfected with miR-101 mimics, VEGF-C siRNA, or co-transfected with miR-101 mimics and VEGF-C plasmid. Magnification, x100. Control, untransfected ICC-9810 cells. **P<0.01 vs. the control cells.

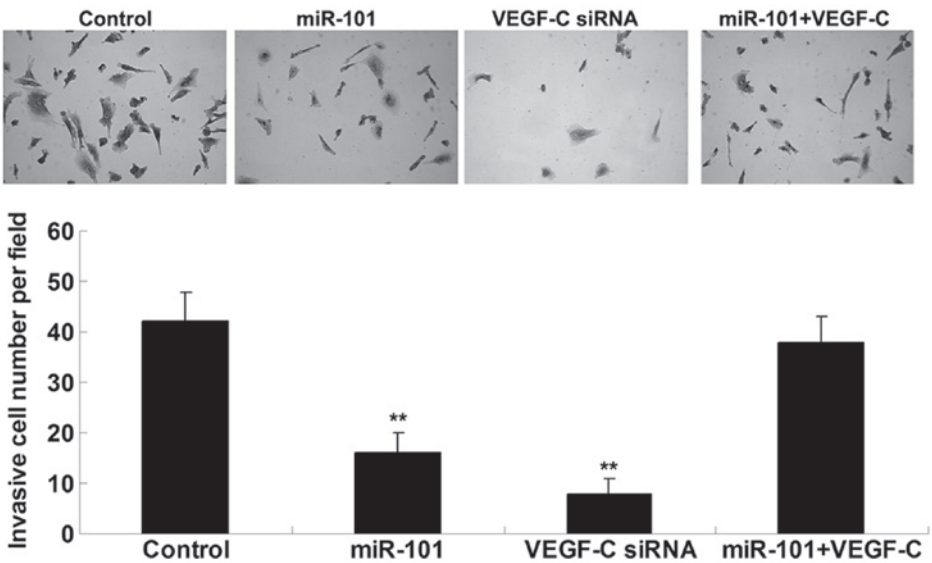

Figure 4. Transwell assay was performed to determine the invasive capacity of ICC-9810 intrahepatic cholangiocarcinoma cells transfected with microRNA (miR)-101 mimics, vascular endothelial growth factor (VEGF)-C small interfering (si)RNA, or co-transfected with miR-101 mimics and VEGF-C plasmid. Magnification, x100. Control, untransfected ICC-9810 cells. **P<0.01 vs. the control cells.

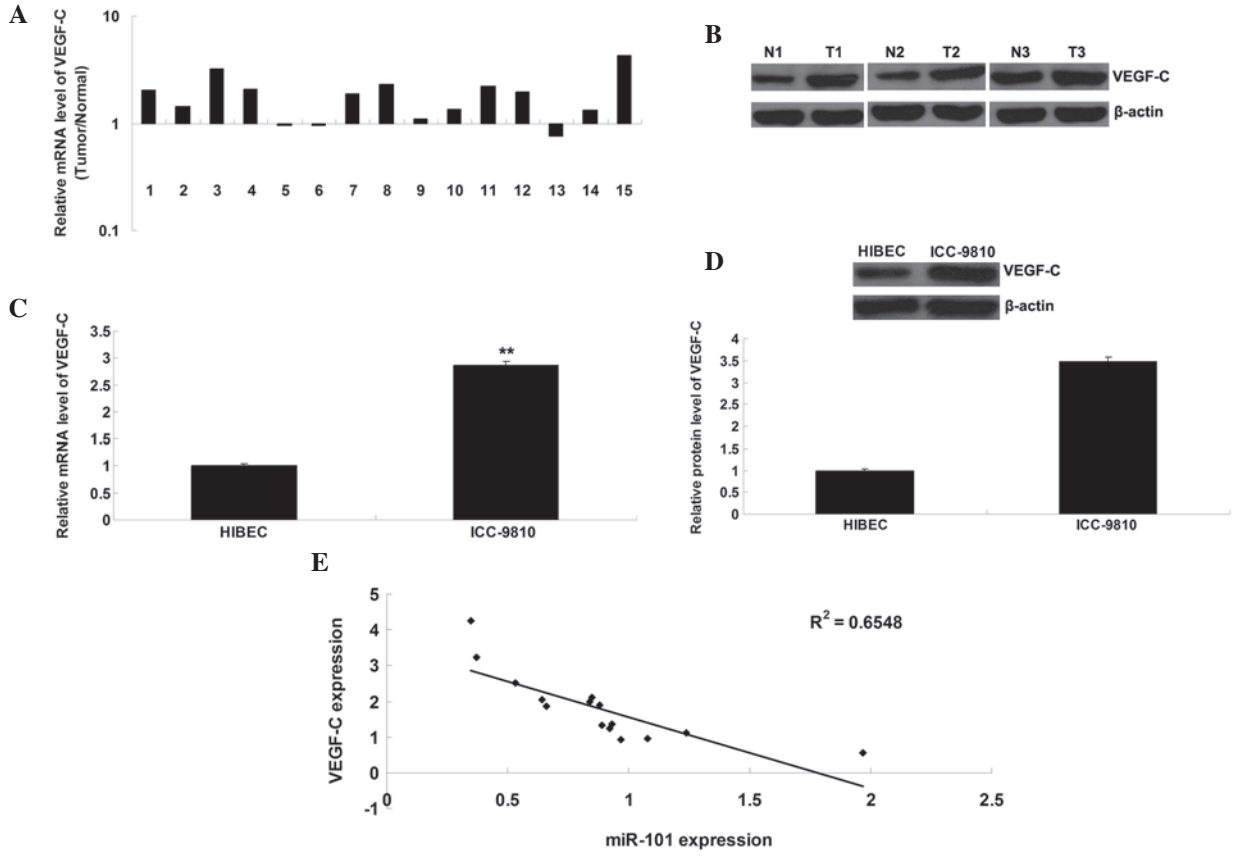

Figure 5. (A) Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to determine the relative mRNA expression levels of vascular endothelial growth factor (VEGF)-C in intrahepatic cholangiocarcinoma (ICC) tumor tissue samples and matched normal adjacent tissue samples. (B) Western blotting was performed to determine the relative protein expression levels of VEGF-C in ICC tissue samples and matched normal adjacent tissue samples. Three representative results are shown. (C) RT-qPCR was used to determine the relative mRNA expression levels of VEGF-C in the ICC-9810 human ICC cell line and in the normal human intrahepatic biliary epithelial cells (HIBEC). **P<0.01 vs. HIBECs. (D) Western blotting was performed to determine the relative protein expression levels of VEGF-C in the ICC-9810 human ICC cell line and normal HIBECs. **P<0.01 vs. HIBECs. (E) An inverse correlation was detected between miR-101 and VEGF-C expression.

The present study is the first, to the best of our knowledge, to detect a marked downregulation of miR-101 expression in ICC tissue and cell lines, suggesting that miR-101 may also be involved in ICC progression.

A previous study demonstrated that miR-101 has a suppressive role in the regulation of cancer metastasis (22). Zhang *et al* (23) reported that miR-101 was able to inhibit the metastasis of osteosarcoma cells via downregulation of enhancer of zeste homolog 2 expression (23). Wang *et al* (24) demonstrated that miR-101 may suppress the migration and invasion of thyroid cancer cells by directly targeting Rac1. The present study demonstrated that restoration of miR-101 expression markedly inhibited the migration and invasion of ICC-9810 cells, thus suggesting that miR-101 may also exert an inhibitory effect on ICC metastasis.

VEGF-C is an important member of the VEGF family, which has been demonstrated to participate in angiogenesis (25). VEGF-C has a role in the regulation of proliferation and motility of endothelial cells, and can also affect vascular permeability (26). The present study identified VEGF-C as a novel target gene of miR-101, and the expression of VEGF-C was shown to be negatively regulated by miR-101. VEGF-C has previously been shown to be involved in the development and progression of various types of human cancer (27). In addition, VEGF-C is associated with lymph node metastasis in numerous types of human malignancy, and knockdown of VEGF-C expression notably inhibits cancer metastases (27).

VEGF-C is also associated with the progression of ICC (4,28). Park *et al* (4) examined the expression of VEGF-C in surgical specimens from 36 patients with ICC, and determined that VEGF-C was frequently upregulated in ICC tissue. Furthermore, VEGF-C expression was significantly correlated with lymph node metastasis, the presence of positive surgical margins and poor survival rate, thus suggesting that strong VEGF-C expression may be an independent factor that indicates poor prognosis (4). Shi *et al* (28) demonstrated that Dickkopf-related protein 1 enhanced tumor cell invasion and promoted the lymph node metastasis of ICC via upregulation of VEGF-C. The present study reported that siRNA-mediated VEGF-C downregulation markedly inhibited the migration and invasion of ICC-9810 cells, whereas upregulation of VEGF-C attenuated the suppressive effects of miR-101 overexpression on ICC-9810 cell migration and invasion. These findings indicated that VEGF-C may be involved in miR-101-mediated migration and invasion of ICC cells.

In conclusion, the results of the present study suggest that miR-101 has an inhibitory role in the regulation of ICC cell migration and invasion, at least in part, via direct inhibition of

VEGF-C expression. Therefore, miR-101/VEGF-C signaling may serve as a potential diagnostic and therapeutic target for ICC.

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