European Review for Medical and Pharmacological Sciences 2020; 24: 2321-2334

LncRNA DANCR affected cell growth, EMT and angiogenesis by sponging miR-345-5p through modulating Twist1 in cholangiocarcinoma

C.-Y. ZHU[1], C.-R. FAN[1], Y.-L. ZHANG[1], Q.-X. SUN[1], M.-J. YAN[1], W. WEI[1], G.-F. LIU[1], J.-J. LIU[2]

[1]Department of Abdominal Surgery, Linyi Cancer Hospital, Linyi, Shandong, China
[2]Department of Internal Medicine, Linyi Cancer Hospital, Linyi, Shandong, China

Abstract. – OBJECTIVE: LncRNA DANCR has been reported to play an important role in various cancers. Therefore, this study aimed at exploring the function and regulatory mechanism of DANCR in Cholangiocarcinoma (CCA).

PATIENTS AND METHODS: qRT-PCR was used to measure the expression of DANCR, miR-345-5p in tissues and cells. Western blot was applied to measure the protein expression of Twist, N-cadherin, Vimentin, E-cadherin, VEGF-A, VEGF-C, PCNA and C-caspase 3. The relationship between DANCR and miR-345-5p was determined by luciferase reporter assay. MTT assay and flow cytometry were used to assess cell proliferation and apoptosis, respectively. Transwell assay was performed to detect cell invasion and migration.

RESULTS: We found that the expression of DANCR was significantly induced in CCA tissues and cells. Inhibition of DANCR remarkably suppressed CCA cell proliferation, migration, invasion, EMT and angiogenesis as well as induced cell apoptosis *in vitro* and *in vivo*. Luciferase reporter assay determined that DANCR directly targeted miR-345-5p and Twist1 was a target mRNA of miR-345-5p. Otherwise, miR-345-5p down-expression partially reversed the effect induced by the suppression of DANCR in CCA. Moreover, the suppressive effects of high miR-345-5p expression on CCA cells were reversed by improving Twist1 expression.

CONCLUSIONS: In this study, we verified that LncRNA DANCR affected cell proliferation, migration, invasion, angiogenesis, epithelial-mesenchymal transition (EMT) and induced apoptosis through modulating miR-345-5p/Twist1 axis in Cholangiocarcinoma.

Key Words:
Cholangiocarcinoma, LncRNA DANCR, MiR-345-5p, Twsit1, Cell growth, Angiogenesis, EMT.

Introduction

Cholangiocarcinoma (CCA) is a common primary hepatobiliary carcinoma which is composed of mutant biliary medial epithelial cells [1]. Surgery is one of the primary means for treating CAA, but the recurrence rate of CCA is high and the prognosis is poor [2,3]. Therefore, there is an urgent need to understand the pathogenesis of CCA and find new therapeutic targets for CCA patients.

LncRNAs play roles in CAA proliferation, which are closely involved in cell proliferation, invasion, migration, apoptosis, angiogenesis and chemosensitivity[4-9]. LncRNAs has been verified to sponge miRNA in 3'UTR to modulate the biological behavior of tumor cells[10-14]. Consistently, lncRNA CASC2 inhibited cell viability and induced cell apoptosis in hepatocellular carcinoma cells through targeting miR-24-3p[15]. lncRNA MALAT1 modulated CCA proliferation, migration and invasion through interacting with miR-204[16]. Zhang et al[17] reported that CCAT1 contributed to cell migration, invasion and EMT in CAA through inhibiting miR-152 expression. In our study, we found that DANCR expression was significantly induced in CCA tissues compared with normal tissues in agreement with previous study[18]. However, the function of DANCR in CCA has not been fully understood.

In this study, we explored the role of DANCR in CCA and our results showed that DANCR was associated with cell proliferation, invasion, migration, epithelial-mesenchymal transition (EMT) and angiogenesis in CCA. Bioinformatics predicts that miR-345-5p is a potential target miRNA for DANCR, thus we aimed to investi-

Corresponding Author: Jinju Liu, MD; e-mail: qvyycp@163.com

gate the regulatory mechanism of DANCR and miR-345-5p in CCA.

Patients and Methods

Patients and Samples

A total of 40 CAA tissues and 40 matched tumor-adjacent tissues were obtained from 40 CAA patients at Linyi Cancer Hospital (Linyi, Shandong, China). The written informed consent was signed by all participants. This study obained the approval of the Research Ethic Committee of Linyi Cancer Hospital.

Cell Cultured and Transfection

CAA cell lines (HuH28, HuCCT1 and SG231) and normal cell line (H69) were purchased from the Institute of Biochemistry and Cell Biology (Chinese Academy of Science, Shanghai, China). Then, cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) at 37°C with 5% of CO_2.

Si-NC, si-DANCR, pcDNA-DANCR, pcDNA-NC, anti-NC, anti-345-5p, sh-NC, sh-DANCR, vector and pcDNA-Twist1 (Twist1) plasmid were purchased from GenePhama (Shanghai, China). All miRNA inhibitors and plasmids were transfected into HuCCT1 and SG231 cells using Lipofectamine 2000 (Invitrogen).

qRT-PCR

Total RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For miRNA, cDNA was reverse transcribed using TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). For mRNA, cDNA was reverse transcribed using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). Real-time PCR was performed using a SYBR premix ExTaq kit (TaKaRa, Dalian, China) in an ABI 7500 system (Applied Biosystems). GAPDH and U6 were employed as reference gene for mRNA and miRNA, respectively. The expression of miRNA and mRNA was calculated using the $2^{-\Delta\Delta CT}$ method. Primer sequences are as follows: GAPDH forward: 5'-AACGTGTCAGTGGTGGACCTG-3', and reverse: 5'-AGTGGGTGTCGCTGTTGAAGT-3'; DANCR Forward: 5'-CGGAGGTGGATTCTGTTAGG-3', and reverse: 5'-TCGGTGTAGCAAGTCTGGTG-3' Twist1 Forward: 5'-CCACTGAAAGGAAAGGCATC-3', and reverse: 5'-CTATGGTTTTGCAGGCCAGT-3' U6 forward: 5'-CTCGCTTCGGCAGCACA-3', and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. miR-345-5p looped reverse primer, 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGAGCCCTG-3'.

Western Blot

Cells were washed with Tris-buffered saline (TBS) and added with the RIPA buffer (Thermo-Fisher Scientific, Inc., Waltham, MA, USA). Total proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Millipore, Billerica, MA, USA). Next, membranes were incubated with primary antibodies anti-Twist1, anti-VEGF-A, anti-VEGF-C, anti-N-cadherin, anti-Vimentin, anti-E-cadherin and anti-GADPH (1:1500, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 4°C overnight. After washing membranes with TBST, they were incubated with peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG (1:1000, Santa Cruz Biotechnology Inc.). The blot was measured using the Pierce™ ECL Western blotting substrate (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's recommendation.

Cell Proliferation

Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay kit (Abcam, Cambridge, MA, USA) according to the manufacturer's protocol. Cells were seeded into 96-well plates at a density of $2x10^3$ cells per well. After that, 50 ul MTT solution was added into each well and the plate was incubated at 37°C with 5% of CO_2 for 4 h. 150 μL MTT solvent (4 mM HCl, 0.1% NP40 in isopropanol) were added into each well and following incubation for 3 h at 37°C with 5% of CO_2. The optical density was determined using a spectrophotometric microplate reader (Beyotime Institute of Biotechnology, Shanghai, China) at the wavelength of 450 nm.

Cell Migration and Invasion

Cell invasion was detected using transwell invasion assay. The transwell (Corning, NY, USA) and Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) were used to detect the cell invasion following manufacturer's protocol. Cell migration was detected using Transwell migration assay. Cell invasion was detected using transwell without Matrigel. Cells were seeded in the upper chamber. After they were incubated at 37°C with 5% of CO_2 for 24 h, invasion cell numbers through the Matrigel was counted and migration cell numbers were counted using a Leica DM3000 microscope (Leica, Wetzlar, Germany).

Cell Apoptosis

The Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences) was used to detect cell apoptosis according to the manufacturer's instructions. After cells were transfected for 48 h, they were digested with trypsin and washed with PBS. Then, the binding buffer was added to resuspend the cells followed with adding the Annexin VFITC and propidium iodide (PI). Flow cytometry was performed to assess cell apoptosis.

Luciferase Reporter Assay

The wild type (DANCR-wt) and mutant type (DANCR-mut) were amplified by PCR and inserted into pMIR-REPORT™ (Thermo-Fisher Scientific) according to the manufacturer's protocols. Then DANCR-wt or DANCR-mut was co-transfected with NC or miR-345-5p mimics (miR-345-5p) into HuCCT1 and SG231 cells using Lipofectamine 2000 (Invitrogen). The wild type (Twist1-wt) and mutant type (Twist1-mut) were amplified by PCR and inserted into pMIR-REPORT™ (Thermo-Fisher Scientific) according to the manufacturer's protocols. Then Twist1-wt or Twist1-mut was co-transfected with NC or miR-345-5p mimics (miR-345-5p) into HuCCT1 and SG231 cells using Lipofectamine 2000 (Invitrogen).

Tumor Formation in Nude Mice Models

HuCCT1 cells stably with DANCR down-expression (sh-DANCR) or the negative control (sh-NC) were subcutaneously injected into the same athymic male BALB/c nude mice at 4-5 weeks of age. Each group has six mice. After injection for 5 weeks, the mice were sacrificed, and the growth of tumor was measured. The tumor tissues were stored at -80°C for the analysis of qRT-PCR and Western blot. All animal experiments were approved by the Clinical Research Ethics Committees of Linyi Cancer Hospital.

Statistical Analysis

The analysis of the results was displayed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). All data were presented as mean ± SD (standard deviation). All comparisons were analyzed using Student's *t*-tests. Statistical significance was defined as $*p<0.05$.

Results

DANCR Expressed High in CCA Tissues and Cells

To explore the biological function of DANCR in the pathogenesis of CCA, we firstly detected DANCR expression in 35 pairs of CCA tissues and adjacent normal tissues using qRT-PCR. The analysis showed that the expression of DANCR was significantly induced in CAA tissues in comparison to adjacent normal tissues (Figure 1A). Then we measured the expression of DANCR in CAA cell lines (HuH28, HuCCT1 and SG231) and normal cell line (H69) for further identification. The results showed that DANCR expression in CAA cells lines was significantly more than that in normal cell lines (Figure 1B). Due to the least expression, we selected HuCCT1 and G231 cell line for the next experiments. More than that, compared with patients with low expression of lncRNA DANCR, the survival rate of patients with high expression of lncRNA DNACR was significantly reduced (Figure 1C). In addition, analysis of clinical case characteristics showed that the expression of lncRNA DANCR was significantly positively correlated with tumor size, TNM state and lymph node metastasis in CCA (Table I). These findings showed that perhaps DANCR was associated with the pathogenesis and metastasis of CCA.

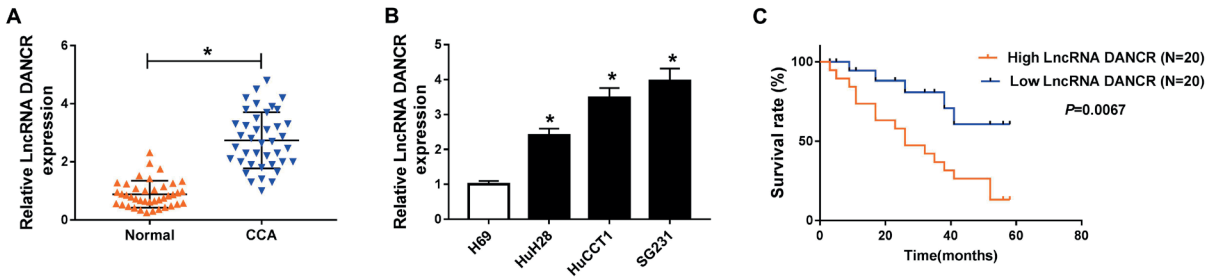

Figure 1. DANCR expressed high in CCA tissues and cells. **(A)** qRT-PCR was used to detect the expression of DNACR in CAA tissues and adjacent normal tissues. **(B)** qRT-PCR was used to detect the expression of DNACR in CAA cell lines (HuH28, HuCCT1 and SG231) and normal cell line (H69). **(C)** Kaplan-Meier survival curves of CCA for patients with high (n=20) and low (n=20) lncRNA DANCR expression. $*p<0.05$.

Table I. Correlation between LncRNA DANCR expression and clinicopathological parameters of Cholangiocarcinoma patients (n=40).

Clinicopathologic feature	n	LncRNA DANCR		*p*-value
		High	Low	
Age				0.3422
≥ 60	21	9	12	
< 60	19	11	8	
Gender				0.5250
Man	22	12	10	
Woman	18	8	10	
Tumor site				0.7233
Intrahepatic	11	5	6	
Extrahepatic	29	15	14	
Tumor size				0.0252
≥ 3 cm	23	15	8	
< 3 cm	17	5	12	
TNM state				0.0058
I-II	12	2	10	
III-IV	28	18	10	
Lymph node metastasis				0.0033
Negative	15	3	12	
Positive	25	17	8	

Down-expression of DANCR Inhibited Cell Proliferation, Migration, Invasion, EMT, Angiogenesis and Induced Apoptosis in CAA Cells

To further investigate the function of DANCR in CAA, we down-expressed DANCR in HuCCT1 and SG231cells (Figure 2A). The analysis of MTT assay displayed that si-DANCR transfection significantly inhibited cell proliferation compared with NC and control in HuCCT1 and SG231cells (Figure 2B). Flow cytometry analyzed that cell apoptosis in si-DANCR groups was remarkably higher than that in control and NC groups in HuCCT1 and SG231cells (Figure 2C). Transwell assay was applied to determine CAA cell invasion and migration. The results showed that cell migration and invasion of si-DANCR group observably decreased compared with control and NC groups in HuCCT1 and SG231cells (Figure 2D and 2E). VEGF-A and VEGF-C are important regulatory factors in angiogenesis. Moreover, si-DANCR transfection inhibited the protein level of VEGF-A and VEGF-C in HuCCT1 and SG231cells (Figure 2Fa-b). As shown in Figure 2G, si-DANCR transfection significantly inhibited N-cadherin and Vimentin protein level and increased E-cadherin protein level in HuCCT1 and SG231 cells. Therefore, these data revealed that down-expression of DANCR inhibited cell proliferation, migration, invasion, EMT, angiogenesis and induced apoptosis in CAA cells.

miR-345-5p Was a Target miRNA of DANCR

To further explore the regulatory mechanism of DANCR, we predicted the target miRNA of DANCR using ENCORI and found that miR-345-5p was a potential target miRNA of DANCR because the 3'UTR of DANCR contained complementary sequences of miR-345-5p (Figure 3A). Luciferase reporter assay was performed to verify the target relationship of between DANCR and miR-345-5p. As shown in Figure 3B, luciferase activity was significantly decreased when the miR-345-5p bound to the DANCR-wt, but there was no change in luciferase activity when combined with DANCR-mut in HuCCT1 and SG231 cells. In addition, we detected the expression of miR-345-5p in HuCCT1 and SG231 cells transfected with pcDNA-DANCR and anti- DANCR, respectively. The results showed that enhanced DANCR expression inhibited miR-345-5p expression, whereas reduced its expression enhancing miR-345-5p expression in HuCCT1 and SG231 cells (Figure 3C). Furthermore, the analysis of qRT-PCR determined that the expression of miR-345-5p was down-regulated in CCA tissues and cells (Figure 3D and 3E). In addition, Pearson's related analysis showed that the expression of DANCR was negatively associated with miR-345-5p expression in CCA tissues (Figure 3F). Taken together, these results indicated that DANCR regulated the expression of miR-345-5p and miR-345-5p was a target miRNA of DANCR in CAA cells.

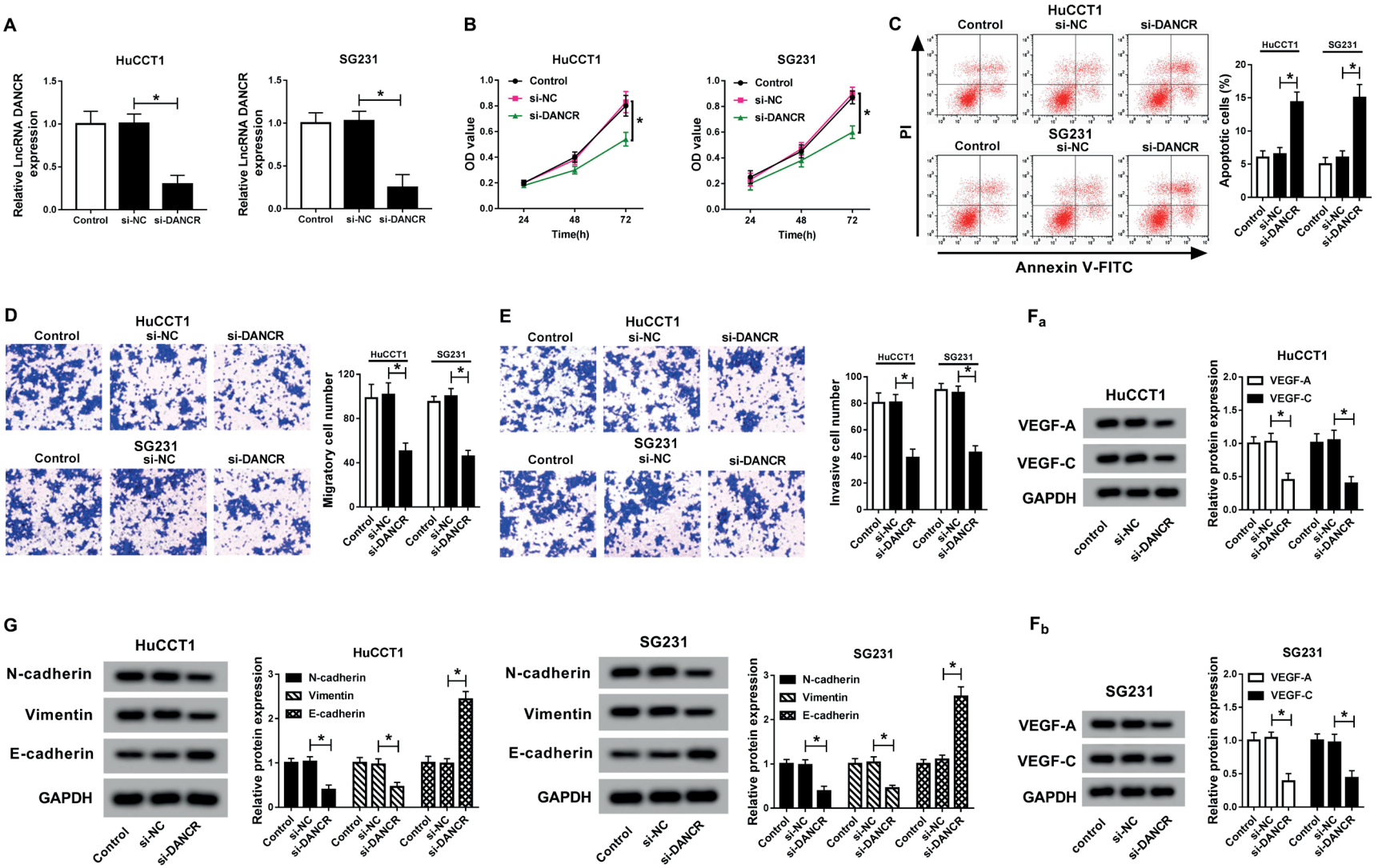

Figure 2. Down-expression of DANCR inhibited cell proliferation, migration, invasion, EMT, angiogenesis and induced apoptosis in CAA cells. **(A)** qRT-PCR was used to detect the expression of DNACR in control, si-NC and si-DNACR groups in HuCCT 1 and SG231 cells. **(B)** MTT assay was applied to measure cell proliferation in control, si-NC and si-DNACR groups in HuCCT1 and SG231 cells. **(C)** Flow cytometry was performed to detected cell apoptosis in control, si-NC and si-DNACR groups in HuCCT1 and SG231 cells. **(D** and **E)** Transwell migration and invasion assays were used to measured cell migration **(D)** and invasion **(E)** in control, si-NC and si-DNACR groups in HuCCT1 and SG231 cells (100x). **(F)** Western blot was applied to measure the protein expression of VEGF-A and VEGF-C in control, si-NC and si-DNACR groups in HuCCT1 and SG231 cells. **(G)** Western blot was used to detect the protein expression of N-cadherin, Vimentin and E-cadherin in control, si-NC and si-DNACR groups in HuCCT1 and SG231 cells. *$p< 0.05$.

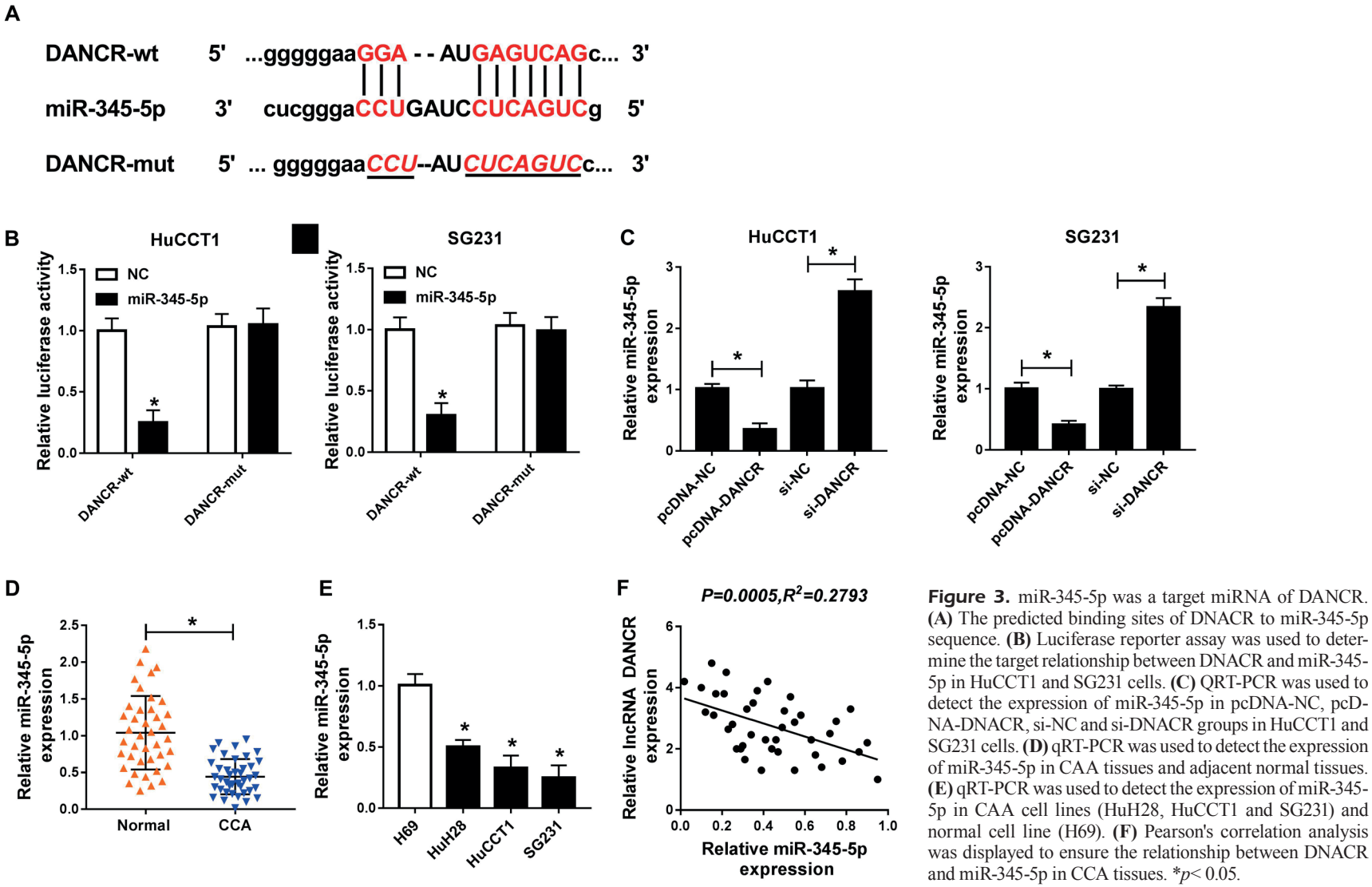

Figure 3. miR-345-5p was a target miRNA of DANCR. **(A)** The predicted binding sites of DNACR to miR-345-5p sequence. **(B)** Luciferase reporter assay was used to determine the target relationship between DNACR and miR-345-5p in HuCCT1 and SG231 cells. **(C)** QRT-PCR was used to detect the expression of miR-345-5p in pcDNA-NC, pcDNA-DNACR, si-NC and si-DNACR groups in HuCCT1 and SG231 cells. **(D)** qRT-PCR was used to detect the expression of miR-345-5p in CAA tissues and adjacent normal tissues. **(E)** qRT-PCR was used to detect the expression of miR-345-5p in CAA cell lines (HuH28, HuCCT1 and SG231) and normal cell line (H69). **(F)** Pearson's correlation analysis was displayed to ensure the relationship between DNACR and miR-345-5p in CCA tissues. *$p< 0.05$.

DANCR Regulated Cell Progression, EMT and Angiogenesis Through Targeting MiR-345-5p in CAA Cells

To prove whether DANCR affected cellular processes by regulating miR-345-5p expression, si-DANCR was co-transfected with anti-miR-345-5p or anti-NC into HuCCT1 and SG231 cells. Next, we detected the expression of miR-345-5p in si-NC, si-DANCR, si-DANCR + anti-NC and si-DANCR+anti-miR-345-5p groups in HuCCT1 and SG231 cells. As expected, the expression of miR-345-5p was significantly induced by si-DANCR transfection, which was reversed by inhibiting miR-345-5p expression in HuCCT1 and SG231 cells (Figure 4A). Additionally, cell proliferation, migration and invasion of si-DANCR groups, was significantly enhanced through anti-miR-345-5p transfection (Figure 4B, 4D and 4E), but cell apoptosis of si-DANCR groups was significantly inhibited through anti-miR-345-5p transfection (Figure 4C) in HuCCT1 and SG231 cells. More than that, the protein of VEGF-A and VEGF-C was decreased by downregulating DANCR, which was impaired by inhibition of miR-345-5p in HuCCT1 and SG231 cells (Figure 4F). More than that, si-DANCR transfection inhibited N-cadherin and Vimentin protein and induced E-cadherin protein, which was reversed by reducing miR-345-5p expression. In conclusion, these results determined that DANCR regulated cell progression, EMT and angiogenesis through targeting miR-345-5p in CAA cells.

MiR-345-5p Directly Targeted Twist1 In CAA Cells

To further determine the regulatory mechanism of DNACR and miR-345-5p, Twist was predicted as a potential mRNA of miR-345-5p by using ENCORI (Figure 5A). Luciferase reporter assay showed that when the miR-354-5p binds to the Twist1-wt 3'UTR, luciferase activity was significantly decreased in HuCCT1 and SG231 cells (Figure 5B). RIP assay also showed that the relative enrichment of Twist was significantly increased in miR-345-5p groups compared with that in NC group in HuCCT1 and SG231 cells (Figure 5C). Interestingly, Twist1 expression was inhibited by miR-345-3p transfection, which was reversed by upregulating DANCR expression in HuCCT1 and SG231 cells (Figure 5D). Moreover, the mRNA and protein expression of Twist1 was remarkably enhanced in CCA tissues and cells compared with Normal groups (Figure 5E, F and G). In addition, we found that the expression of Twist1 was negatively correlated with the expression of miR-345-5p and positively correlated with the expression of DANCR in CAA tissues (Figure 5H and 5I). These results showed that Twist1 was a target of miR-345-3p and involved in the regulatory mechanism of DANCR/miR-345-3p axis in CCA.

Upregulation of Twist1 Reversed the Effects of High MiR-345-5p Expression on CCA Cells

Next, the rescue experiment was applied to determine the relationship between miR-345-5p and Twist1 in CCA. As shown in Figure 6A, the expression of Twist1 was inhibited by miR-345-5p transfection, while the promotion of Twist1 could reverse its effects in HuCCT1 and SG231 cells. Moreover, upregulated Twist1 expression improved cell proliferation inhibited by overexpression of mIR345-5p in HuCCT1 and SG231 cells (Figure 6B). Cell apoptosis was induced by miR-345-5p transfection, which was reversed by high expression of Twist1 in cells (Figure 6C). Otherwise, the capacity of cell invasive and migration reduced by overexpressing miR-345-5p was increased by enhancing Twist1 expression (Figure 6D and 6E). More than that, the protein expression of VEGF-A, VEGF-C, N-cadherin and Vimentin was obviously suppressed while the protein expression of E-cadherin was increased via increasing miR-345-5p. However, these effects were alleviated by improving Twist1 expression in cells (Figure 6F and 6G). Thus, these results showed that miR-345-3p affected cell progression, EMT and angiogenesis through regulating Twist1 expression in CCA.

Silenced DANCR Expression Inhibited Tumor Growth In Vivo

To further investigate the function of DANCR in tumor of CCA, sh-NC or sh-DANCR was transfected into HuCCT1 cells and then injected into the mice. As shown in Figure 7A and 7B, we found that tumor volume and weight of sh-DANCR transfection were significantly inhibited compared with that of sh-NC transfection. Additionally, sh-DANCR transfection decreased DANCR expression (Figure 7C). More than that, the protein expression of VEGF-A, VEGF-C and PCNA was inhibited and C-caspase 3 was induced by suppression of DANCR (Figure 7D). Therefore, these data suggested that silenced DANCR expression inhibited tumor growth *in vivo*.

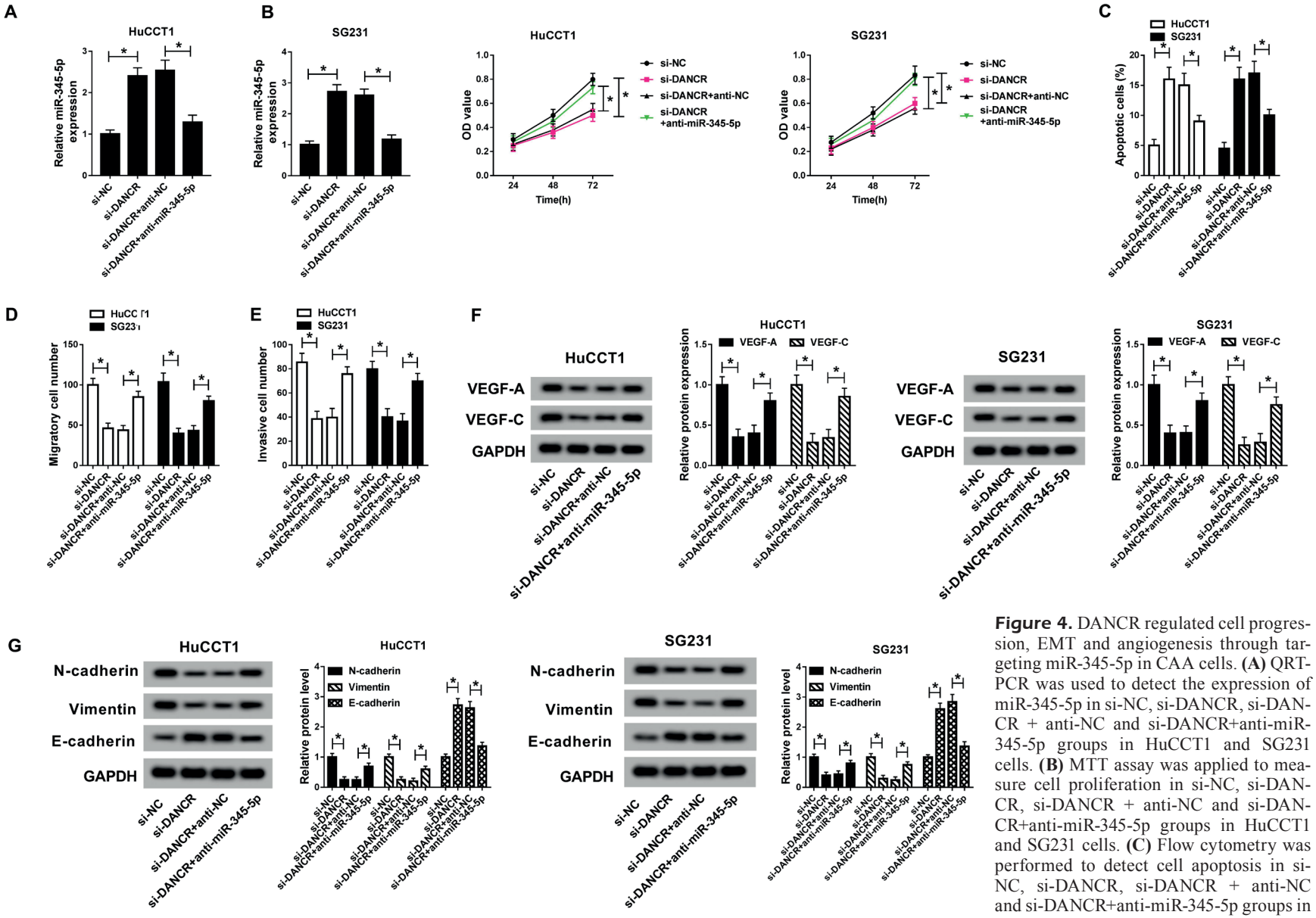

Figure 4. DANCR regulated cell progression, EMT and angiogenesis through targeting miR-345-5p in CAA cells. **(A)** QRT-PCR was used to detect the expression of miR-345-5p in si-NC, si-DANCR, si-DANCR + anti-NC and si-DANCR+anti-miR-345-5p groups in HuCCT1 and SG231 cells. **(B)** MTT assay was applied to measure cell proliferation in si-NC, si-DANCR, si-DANCR + anti-NC and si-DANCR+anti-miR-345-5p groups in HuCCT1 and SG231 cells. **(C)** Flow cytometry was performed to detect cell apoptosis in si-NC, si-DANCR, si-DANCR + anti-NC and si-DANCR+anti-miR-345-5p groups in HuCCT1 and SG231 cells. **(D** and **E)** Transwell migration and invasion assays were used to measure cell migration **(D)** and invasion **(E)** in si-NC, si-DANCR, si-DANCR + anti-NC and si-DANCR+anti-miR-345-5p groups in HuCCT1 and SG231 cells. **(F)** Western blot was used to detect the protein expression of VEGF-A and VEGF-C in si-NC, si-DANCR, si-DANCR + anti-NC and si-DANCR+anti-miR-345-5p groups in HuCCT1 and SG231 cells. **(G)** Western blot was used to detect the protein expression of N-cadherin, Vimentin and E-cadherin in si-NC, si-DANCR, si-DANCR + anti-NC and si-DANCR+anti-miR-345-5p groups in HuCCT1 and SG231 cells. *$p < 0.05$.

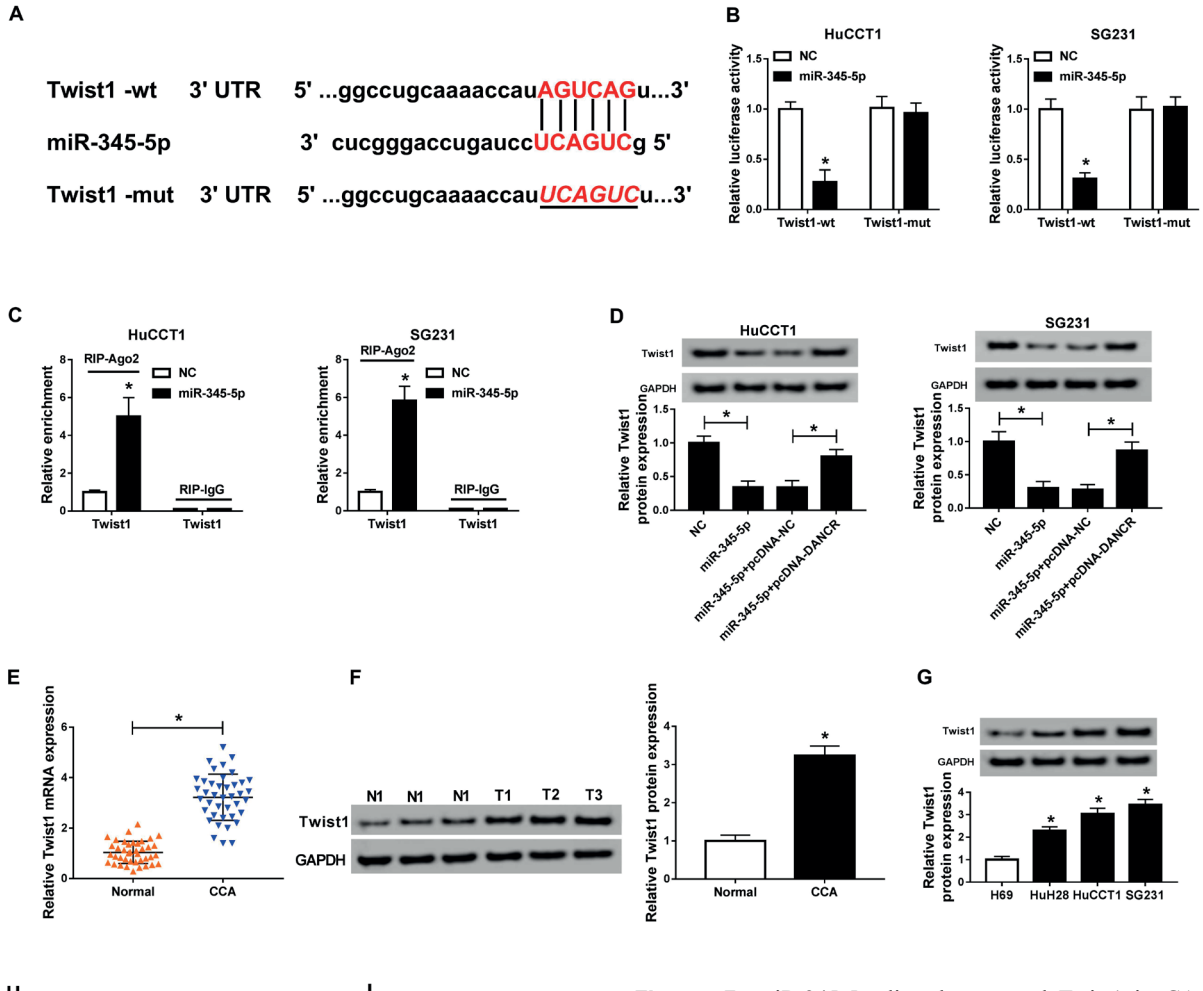

Figure 5. miR-345-5p directly targeted Twist1 in CAA cells. **(A)** The predicted binding sites of Twist1 to miR-345-5p sequence. **(B)** Luciferase reporter assay was used to determine the target relationship between Twist1 and miR-345-5p in HuCCT1 and SG231 cells. **(C)** RIP assay was detected to measure the relative enrichment of Twist1 in CCA cells. **(D)** Western blot was used to detect the expression of miR-345-5p in NC, miR-345-5p, miR-345-5p+pcDNA-NA and miR-345-5p+pcDNA-DANCR groups in HuCCT1 and SG231 cells. **(E** and **F)** qRT-PCR and Western blot were used to detect the mRNA and protein expression of Twist1 in CAA tissues and adjacent normal tissues. **(G)** qRT-PCR was used to detect the expression of Twist1 in CAA cell lines (HuH28, HuCCT1 and SG231) and normal cell line (H69). **(H)** Pearson's correlation analysis was displayed to ensure the relationship between Twist and miR-345-5p in CCA tissues. **(I)** Pearson's correlation analysis was displayed to ensure the relationship between DNACR and Twist1 in CCA tissues. *$p < 0.05$.

Discussion

LncRNAs and miRNAs have been demonstrated to participate in cell growth, EMT and angiogenesis in multiple cancers, such as gastric cancer, thyroid cancer, breast cancer, bladder cancer and non-small cell lung cancer[19-23]. Importantly, lncRNA MALAT1, FOXM1, miR-15a/16, miR-342-3p and miR-542-3p have been verified to be involved in angiogenesis[24-27], which might also be related with cell proliferation, cell cycle and cell growth[28-32]. More than that, lncRNA UCA1 could promote EMT through activating Wnt/beta-catenin signaling pathway[33] and so on. Therefore, lncRNAs and miRNAs were important regulators in cell development and progression.

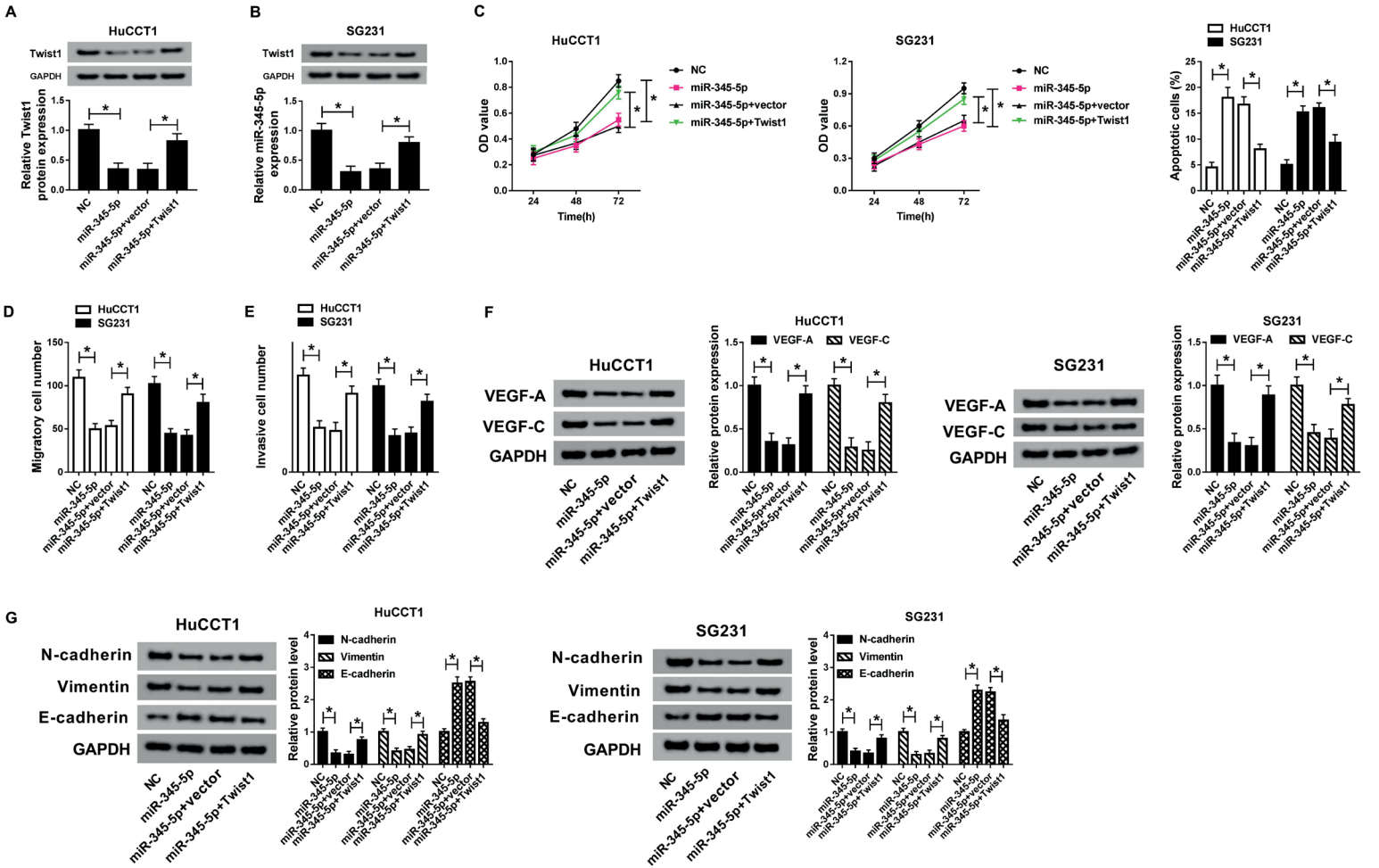

Figure 6. Upregulation of Twist1 reversed the effects of high miR-345-5p expression on CCA cells. **(A)** Western blot was used to detect the expression of miR-345-5p in NC, miR-345-5p, miR-345-5p + vector and s miR-345-5p + Twist1 groups in HuCCT1 and SG231 cells. **(B)** MTT assay was applied to measured cell proliferation in NC, miR-345-5p, miR-345-5p + vector and s miR-345-5p + Twist1 groups in HuCCT1 and SG231 cells. **(C)** Flow cytometry was performed to detect cell apoptosis in NC, miR-345-5p, miR-345-5p + vector and s miR-345-5p + Twist1 groups in HuCCT1 and SG231 cells. **(D** and **E)** Transwell migration and invasion assays were used to measured cell migration **(D)** and invasion **(E)** in NC, miR-345-5p, miR-345-5p + vector and s miR-345-5p + Twist1 groups in HuCCT1 and SG231 cells. **(F)** Western blot was used to detect the protein expression of VEGF-A and VEGF-C in NC, miR-345-5p, miR-345-5p + vector and s miR-345-5p + Twist1 groups in HuCCT1 and SG231 cells. **(G)** Western blot was used to detect the protein expression of N-cadherin, Vimentin and E-cadherin in NC, miR-345-5p, miR-345-5p + vector and s miR-345-5p + Twist1 groups in HuCCT1 and SG231 cells. *$p < 0.05$.

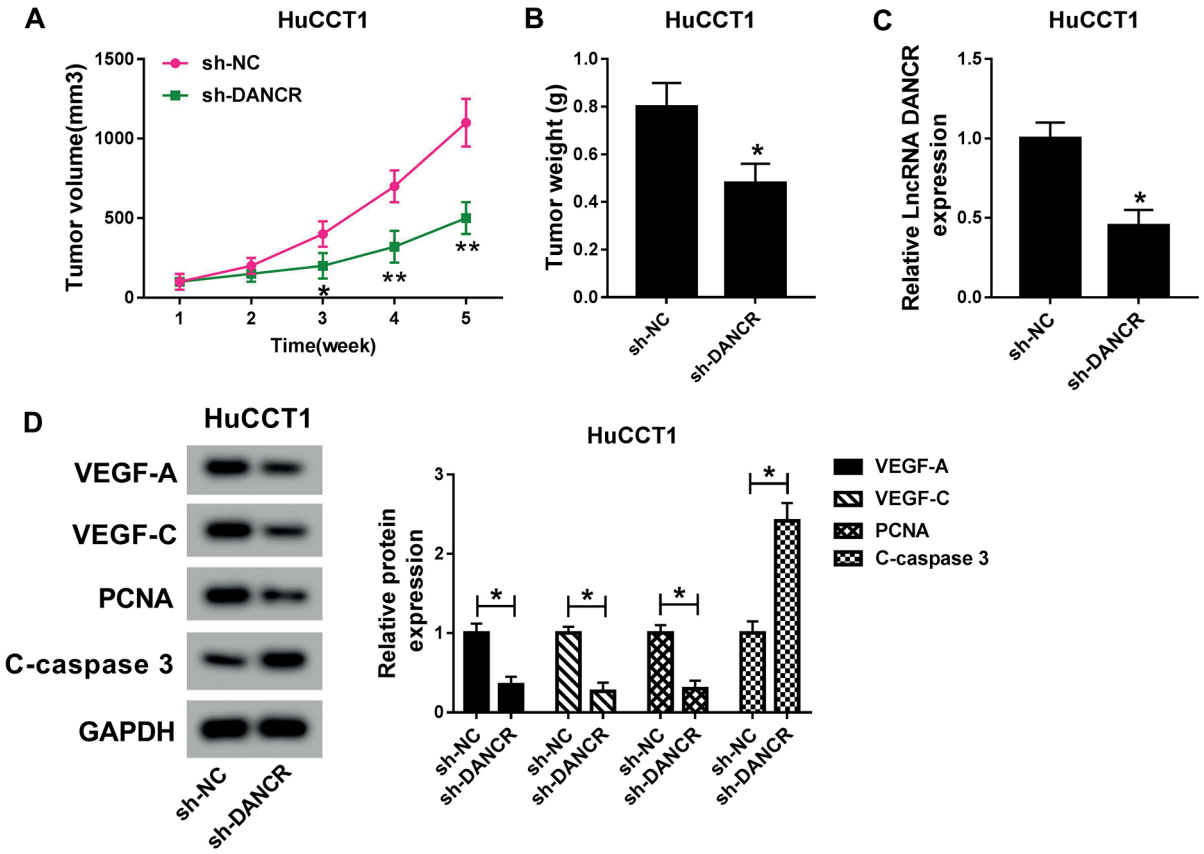

Figure 7. Silenced DANCR expression inhibited tumor growth in vivo. **(A)** Tumor volume was measured in sh-NC and sh-DANCR groups in vivo. **(B)** Tumor weight was measured in sh-NC and sh-DANCR groups in vivo. **(C)** The expression of DANCR was detected in sh-NC and sh-DANCR groups with qRT-PCR. **(D)** The protein expression of VEGF-A, VEGF-C, PCNA and C-caspase 3 was measured in sh-NC and sh-DANCR groups with Western blot. *$p < 0.05$.

In CCA, some lncRNAs and miRNAs have also been verified to be associated with cell proliferation and chemoresistance, such as TUG1, ASAP1-IT1, AFAP1-AS1, CCAT2, miR-199-3p, miR-25[5,34-38]. VEGF-A and VEGF-C have been determined to be upregulated in various cancers and combined with receptors on vascular endothelial cells, directly promoting the formation of blood vessels in tumors and eventually leading to the growth, invasion and metastasis of tumors[39-43]. N-cadherin, Vimentin and E-cadherin are the essential proteins in EMT of cancer cells, reflecting the metabolicness of cancer cells. In this study, DANCR expression was significantly increased in CCA tissues and cells and inhibition of DANCR could inhibit CCA cell proliferation, migration and invasion, and induced cell apoptosis. Moreover, reduced DANCR could inhibit tumor growth *in vivo*. DANCR played an important role in cell progression of various cancers, which was a biomarker in the diagnosis of cancers[44-46]. Of note, we also found that suppressed DANCR expression inhibited EMT and angiogenesis in CCA.

To further investigate the regulatory mechanism of DANCR, luciferase reporter assay was applied to determine that miR-345-5p was a target miRNA of DANCR. More than that, miR-345-5p has been reported to be a tumor suppressor in pancreatic cancer[47]. In this study, we found that miR-345-5p expression was decreased in CCA tissues and cells consistent with Yu et al[48]. Inhibited miR-345-5p expression could reverse the suppressive effects of si-DANCR on cell growth, EMT and angiogenesis in CCA, implying that DANCR regulated CCA cell growth, EMT and angiogenesis through targeting miR-345-5p.

Twist-related protein 1 (TWIST1) is a basic helix-loop-helix transcription factor in humans, which has been associated with cell determina-

tion and differentiation[49]. Twist1, acts as an oncogene, and plays an important role in metastasis and invasion of cancer cells[50-52]. Moreover, Twist1 has been reported to be highly expressed in various cancers and participates in a variety of cell progressions, such as apoptosis, invasion EMT, angiogenesis, inflammation and proliferation[53-55]. Twist1 could enhance the ability of invasive via mesenchymal changes in human glioblastoma[56]. In addition, as a target gene, Twist1 interacted with miRNA to affect cell growth and drug resistance, including miR-720, miR-181a, and miR-381[57-59]. More than that, Twist1 was a target gene of miR-186 in CCA and affects cell progression[60]. In this study, Twist1 was upregulated in CCA tissues and cells, consistent with previous studies[61,62]. Otherwise, overexpression of Twist1 expression could reverse the suppressive effects of miR-345-5p transfection on cell growth, EMT and angiogenesis in CCA, implying that miR-345-5p regulated cell growth via targeting Twist1. Based on the above results, DANCR/ mir-345-5p/Twist1 axis is an essential regulatory pathway in the cellular progression of CCA.

Although our results identified the function and a regulatory network of DANCR in CCA, there must be another regulatory network of DANCR, which has not been fully explored. In other cancers, DANCR has been reported to participate in cell cycle, chemoresistance and tumor development[45,63,64]. However, further investigations of DANCR in CCA are needed to investigate the chemoresistance, prognostic and underlying regulatory mechanism of CCA.

Conclusions

We demonstrated that DANCR was upregulated in CCA tissues and cells and affected cell proliferation, invasion, migration, apoptosis, EMT and angiogenesis by modulating miR-345-5p/ Twist1 axis, providing a new regulatory network and a novel therapy target in CCA.

Conflict of Interests

The Authors declare that they have no conflict of interests.

References

1) Rizvi S, Gores GJ. Pathogenesis, diagnosis, and management of cholangiocarcinoma. Gastroenterology 2013; 145: 1215-1229.
2) Khan SA, Toledano MB, Taylor-Robinson SD. Epidemiology, risk factors, and pathogenesis of cholangiocarcinoma. HPB (Oxford) 2008; 10: 77-82.
3) Sirica AE, Nathanson MH, Gores GJ, Larusso NF. Pathobiology of biliary epithelia and cholangiocarcinoma: proceedings of the Henry M. and Lillian Stratton Basic Research Single-Topic Conference. Hepatology 2008; 48: 2040-2046.
4) Zheng B, Jeong S, Zhu Y, Chen L, Xia Q. miRNA and lncRNA as biomarkers in cholangiocarcinoma (CCA). Oncotarget 2017; 8: 100819-100830.
5) Guo L, Zhou Y, Chen Y, Sun H, Wang Y, Qu Y. LncRNA ASAP1-IT1 positively modulates the development of cholangiocarcinoma via hedgehog signaling pathway. Biomed Pharmacother 2018; 103: 167-173.
6) Lv L, Wei M, Lin P, Chen Z, Gong P, Quan Z, Tang Z. Integrated mRNA and lncRNA expression profiling for exploring metastatic biomarkers of human intrahepatic cholangiocarcinoma. Am J Cancer Res 2017; 7: 688-699.
7) Shi X, Zhang H, Wang M, Xu X, Zhao Y, He R, Zhang M, Zhou M, Li X, Peng F, Shi C, Shen M, Wang X, Guo X, Qin R. LncRNA AFAP1-AS1 promotes growth and metastasis of cholangiocarcinoma cells. Oncotarget 2017; 8: 58394-58404.
8) Xu Y, Jiang X, Cui Y. Upregulated long noncoding RNA PANDAR predicts an unfavorable prognosis and promotes tumorigenesis in cholangiocarcinoma. Onco Targets Ther 2017; 10: 2873-2883.
9) Ayers D. Long non-coding RNAs: novel emergent biomarkers for cancer diagnostics. J Cancer Res 2013; 1: 31-35.
10) Ye Y, Gao X, Yang N. LncRNA ZFAS1 promotes cell migration and invasion of fibroblast-like synoviocytes by suppression of miR-27a in rheumatoid arthritis. Human Cell 2018; 31: 14-21.
11) Zhang J, Li XY, Hu P, Ding YS. lncRNA NORAD contributes to colorectal cancer progression by inhibition of miR-202-5p. Oncol Res 2018 Feb 22. doi: 10.3727/096504018X15190844870055. [Epub ahead of print]
12) Jiang H, Ma R, Zou S, Wang Y, Li Z, Li W. Reconstruction and analysis of the lncRNA–miRNA–mRNA network based on competitive endogenous RNA reveal functional lncRNAs in rheumatoid arthritis. Mol Biosyst 2017; 13: 1182-1192.
13) Liu M, Jia J, Wang X, Liu Y, Wang C, Fan R. Long non-coding RNA HOTAIR promotes cervical cancer progression through regulating BCL2 via targeting miR-143-3p. Cancer Biol Ther 2018; 19: 391-399.
14) Lian Y, Li XY, Tang YY, Yang LT, Li XL, Xiong W. Long non-coding RNAs function as competing endogenous RNAs to regulate cancer progression. Biochem Biophys Res Commun 2016; 519: 901-908.
15) Fan JC, Zeng F, Le YG, Xin L. LncRNA CASC2 inhibited the viability and induced the apoptosis of hepatocellular carcinoma cells through regulating miR-24-3p. J Cell Biochem 2018; 119: 6391-6397.
16) Tan X, Huang Z, Li X. Long non-coding RNA MALAT1 interacts with miR-204 to modulate human

hilar cholangiocarcinoma proliferation, migration and invasion by targeting CXCR4. J Cell Biochem 2017; 118: 3643-3653.

17) Zhang S, Xiao J, Chai Y, Du YY, Liu Z, Huang K, Zhou X, Zhou W. LncRNA-CCAT1 promotes migration, invasion, and EMT in intrahepatic cholangiocarcinoma through suppressing miR-152. Dig Dis Sci 2017; 62: 3050-3058.

18) Wang N, Zhang C, Wang W, Liu J, Yu Y, Li Y, Zhang M, Ge X, Li Q, Miao L. Long noncoding RNA DANCR regulates proliferation and migration by epigenetically silencing FBP1 in tumorigenesis of cholangiocarcinoma. Cell Death Dis 2019; 10: 585.

19) Li L, Zhang L, Zhang Y, Zhou F. Increased expression of LncRNA BANCR is associated with clinical progression and poor prognosis in gastric cancer. Biomed Pharmacother 2015; 72: 109-112.

20) Li Q, Shen W, Li X, Zhang L, Jin X. The lncRNA n340790 accelerates carcinogenesis of thyroid cancer by regulating miR-1254. Am J Transl Res 2017; 9: 2181-2194.

21) Li X, Wang S, Li Z, Long X, Wen L. The lncRNA NEAT1 facilitates cell growth and invasion via the miR-211/HMGA2 axis in breast cancer. Int J Biol Macromol 2017; 105: 346-353.

22) Cheng J, Chen J, Zhang X, Mei H, Wang F, Cai Z. Overexpression of CRNDE promotes the progression of bladder cancer. Biomed Pharmacother 2018; 99: 638-644.

23) Nie W, Ge HJ, Yang XQ, Sun X, Huang H, Tao X, Chen WS, Li B. LncRNA-UCA1 exerts oncogenic functions in non-small cell lung cancer by targeting miR-193a-3p. Cancer Lett 2016; 371: 99-106.

24) Huang JK, Song WH, Lu BY, Huang YB, Dong HM, Ma XK, Zhu ZZ, Zhou R. LncRNA-MALAT1 promotes angiogenesis of thyroid cancer by modulating tumor-associated macrophage FGF2 protein secretion. J Cell Biochem 2017; 118: 4821-4830.

25) Monteiro LJ, Cubillos S, Sanchez M, Acuña-Gallardo S, Venegas P, Herrera V, Lam EW, Varas-Godoy M, Illanes SE. Reduced FOXM1 expression limits trophoblast migration and angiogenesis and is associated with preeclampsia. Reprod Sci 2019; 26: 580-590.

26) Li Y, Zhang B, Li W, Wang L, Yan Z, Li H, Yao Y, Yao R, Xu K, Li Z. MiR-15a/16 regulates the growth of myeloma cells, angiogenesis and antitumor immunity by inhibiting Bcl-2, VEGF-A and IL-17 expression in multiple myeloma. Leuk Res 2016; 49: 73-79.

27) He T, Qi F, Jia L, Wang S, Song N, Guo L, Fu Y, Luo Y. MicroRNA-542-3p inhibits tumor angiogenesis by targeting angiopoietin2. J Pathol 2014; 232: 499-508.

28) Chen L, Yao H, Wang K, Liu X. Long non-coding RNA MALAT1 regulates ZEB1 expression by sponging miR-143-3p and promotes hepatocellular carcinoma progression. J Cell Biochem 2017; 118: 4836-4843.

29) Nakamura S, Hirano I, Okinaka K, Takemura T, Yokota D, Ono T, Shigeno K, Shibata K, Fujisawa S, Ohnishi K. The FOXM1 transcriptional factor promotes the proliferation of leukemia cells through modulation of cell cycle progression in acute myeloid leukemia. Carcinogenesis 2010; 31: 2012-2021.

30) Cai CK, Zhao GY, Tian LY, Liu L, Yan K, Ma YL, Ji ZW, Li XX, Han K, Gao J, Qiu XC, Fan QY, Yang TT, Ma BA. miR-15a and miR-16-1 downregulate CCND1 and induce apoptosis and cell cycle arrest in osteosarcoma. Oncol Rep 2012; 28: 1764-1770.

31) Li XR, Chu HJ, Lv T, Wang L, Kong SF, Dai SZ. miR-342-3p suppresses proliferation, migration and invasion by targeting FOXM1 in human cervical cancer. FEBS Lett 2014; 588: 3298-3307.

32) Shen X, Si Y, Yang Z, Wang Q, Yuan J, Zhang X. MicroRNA-542-3p suppresses cell growth of gastric cancer cells via targeting oncogene astrocyte--elevated gene-1. Med Oncol 2015; 32: 361.

33) Xiao C, Wu CH, Hu HZ. LncRNA UCA1 promotes epithelial-mesenchymal transition (EMT) of breast cancer cells via enhancing Wnt/beta-catenin signaling pathway. Eur Rev Med Pharmacol Sci 2016; 20: 2819-2824.

34) Bing Z, Ye H, Chen J, Di C, Cai C, Chen G, Chen X, Xin H, Tang C, Zeng J. LncRNA TUG1 sponges miR-145 to promote cancer progression and regulate glutamine metabolism via Sirt3/GDH axis. Oncotarget 2017; 8: 113650-113661.

35) Lu X, Zhou C, Li R, Deng Y, Zhao L, Zhai W. Long noncoding RNA AFAP1-AS1 promoted tumor growth and invasion in cholangiocarcinoma. Cell Physiol Biochem 2017; 42: 222-230.

36) Xu Y, Yao Y, Qin W, Zhong X, Jiang X, Cui Y. Long non-coding RNA CCAT2 promotes cholangiocarcinoma cells migration and invasion by induction of epithelial-to-mesenchymal transition. Biomed Pharmacother 2018; 99: 121-127.

37) Li Q, Xia X, Ji J, Ma J, Tao L, Mo L, Chen W. MiR--199a-3p enhances cisplatin sensitivity of cholangiocarcinoma cells by inhibiting mTOR signaling pathway and expression of MDR1. Oncotarget 2017; 8: 33621-33630.

38) Razumilava N, Bronk SF, Smoot RL, Fingas CD, Werneburg NW, Roberts LR, Mott JL. miR-25 targets TRAIL death receptor-4 and promotes apoptosis resistance in cholangiocarcinoma. Hepatology 2012; 55: 465-475.

39) George ML, Tutton MG, Janssen F, Arnaoutz A, Abulafi AM, Eccles SA, Swift RI. VEGF-A, VEGF-C, and VEGF-D in colorectal cancer progression. Neoplasia 2001; 3: 420-427.

40) Tang RF, Itakura J, Aikawa T, Matsuda K, Fujii H, Korc M, Matsumoto Y. Overexpression of lymphangiogenic growth factor VEGF-C in human pancreatic cancer. Pancreas 2001; 22: 285-292.

41) Leek RD, Hunt NC, Landers RJ, Lewis CE, Royds JA, Harris AL. Macrophage infiltration is associated with VEGF and EGFR expression in breast cancer. J Pathol 2000; 190: 430-436.

42) Timoshenko AV, Chakraborty C, Wagner GF, Lala PK. COX-2-mediated stimulation of the lymphangiogenic factor VEGF-C in human breast cancer. Br J Cancer 2006; 94: 1154-1163.

43) Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. FASEB J 1999; 13: 9-22.

44) Fan X, Yuan J, Xie J, Pan Z, Yao X, Sun X, Zhang P, Zhang L. Long non-protein coding RNA DANCR functions as a competing endogenous RNA to regulate osteoarthritis progression via miR-577/SphK2 axis. Biochem Biophys Res Commun 2018; 500: 658-664.

45) Ma Y, Zhou G, Li M, Hu D, Zhang L, Liu P, Lin K. Long noncoding RNA DANCR mediates cisplatin resistance in glioma cells via activating AXL/PI3K/Akt/NF-κB signaling pathway. Neurochem Int 2018; 118: 233-241.

46) Tong X, Gu PC, Xu SZ, Lin XJ. Long non-coding RNA-DANCR in human circulating monocytes: a potential biomarker associated with postmenopausal osteoporosis. Biosci Biotechnol Biochem 2015; 79: 732-737.

47) Mou T, Xie F, Zhong P, Hua H, Lai L, Yang Q, Wang J. MiR-345-5p functions as a tumor suppressor in pancreatic cancer by directly targeting CCL8. Biomed Pharmacother 2019; 111: 891-900.

48) Yu J, Zhang B, Zhang H, Qi Y, Wang Y, Wang W, Wang Y, Wang Y. E2F1-induced upregulation of long non-coding RNA LMCD1-AS1 facilitates cholangiocarcinoma cell progression by regulating miR-345–5p/COL6A3 pathway. Biochem Biophys Res Commun 2019; 512: 150-155.

49) Lee MS, Lowe GN, Strong DD, Wergedal JE, Glackin CA. TWIST, a basic helix-loop-helix transcription factor, can regulate the human osteogenic lineage. J Cell Biochem 1999; 75: 566-577.

50) Martin A, Cano A. Tumorigenesis: Twist1 links EMT to self-renewal. Nat Cell Biol 2010; 12: 924-925.

51) Eckert MA, Santiago-Medina M, Lwin TM, Kim J, Courtneidge SA, Yang J. ADAM12 induction by TWIST1 promotes tumor invasion and metastasis via regulation of invadopodia and focal adhesions. J Cell Sci 2017; 130: 2036-2048.

52) Ma G, He J, Yu Y, Xu Y, Yu X, Martinez J, Lonard DM, Xu J. Tamoxifen inhibits ER-negative breast cancer cell invasion and metastasis by accelerating twist1 degradation. Int J Biol Sci 2015; 11: 618-628.

53) Alexander NR, Tran NL, Rekapally H, Summers CE, Glackin C, Heimark RL. N-cadherin gene expression in prostate carcinoma is modulated by integrin-dependent nuclear translocation of Twist1. Cancer Res 2006; 66: 3365-3369.

54) Sun T, Sun BC, Zhao XL, Zhao N, Dong XY, Che N, Yao Z, Ma YM, Gu Q, Zong WK, Liu ZY. Promotion of tumor cell metastasis and vasculogenic mimicry by way of transcription coactivation by Bcl-2 and Twist1: a study of hepatocellular carcinoma. Hepatology 2011; 54: 1690-1706.

55) Niesner U, Albrecht I, Janke M, Doebis C, Loddenkemper C, Lexberg MH, Eulenburg K, Kreher S, Koeck J, Baumgrass R, Bonhagen K, Kamradt T, Enghard P, Humrich JY, Rutz S, Schulze-Topphoff U, Aktas O, Bartfeld S, Radbruch H, Hegazy AN, Löhning M, Baumgart DC, Duchmann R, Rudwaleit M, Häupl T, Gitelman I, Krenn V, Gruen J, Sieper J, Zeitz M, Wiedenmann B, Zipp F, Hamann A, Janitz M, Scheffold A, Burmester GR, Chang HD, Radbruch A. Autoregulation of Th1-mediated inflammation by twist1. J Exp Med 2008; 205: 1889-1901.

56) Mikheeva SA, Mikheev AM, Petit A, Beyer R, Oxford RG, Khorasani L, Maxwell JP, Glackin CA, Wakimoto H, González-Herrero I, Sánchez-García I, Silber JR, Horner PJ, Rostomily RC. TWIST1 promotes invasion through mesenchymal change in human glioblastoma. Mol Cancer 2010; 9: 194.

57) Li LZ, Zhang CZ, Liu LL, Yi C, Lu SX, Zhou X, Zhang ZJ, Peng YH, Yang YZ, Yun JP. miR-720 inhibits tumor invasion and migration in breast cancer by targeting TWIST1. Carcinogenesis 2014; 35: 469-478.

58) Liu M, Wang J, Huang H, Hou J, Zhang B, Wang A. miR-181a-Twist1 pathway in the chemoresistance of tongue squamous cell carcinoma. Biochem Biophys Res Commun 2013; 441: 364-370.

59) He X, Wei Y, Wang Y, Liu L, Wang W, Li N. MiR-381 functions as a tumor suppressor in colorectal cancer by targeting Twist1. Onco Targets Ther 2016; 9: 1231-1239.

60) Zhang M, Shi B, Zhang K. miR-186 suppresses the progression of cholangiocarcinoma cells through inhibition of Twist1. Oncol Res 2019; 27: 1061-1068.

61) Seol MA, Chu IS, Lee MJ, Yu GR, Cui XD, Cho BH, Ahn EK, Leem SH, Kim IH, Kim DG. Genome-wide expression patterns associated with oncogenesis and sarcomatous transdifferentation of cholangiocarcinoma. BMC Cancer 2011; 11: 78.

62) Li B, Han Q, Zhu Y, Yu Y, Wang J, Jiang X. Down-regulation of miR-214 contributes to intrahepatic cholangiocarcinoma metastasis by targeting Twist. FEBS J 2012; 279: 2393-2398.

63) Tao W, Wang C, Zhu B, Zhang G, Pang D. LncRNA DANCR contributes to tumor progression via targetting miR-216a-5p in breast cancer: lncRNA DANCR contributes to tumor progression. Biosci Rep 2019; 39(4). pii: BSR20181618.

64) Zhang L, Sun X, Chen S, Yang C, Shi B, Zhou L, Zhao J. Long noncoding RNA DANCR regulates miR-1305-Smad 4 axis to promote chondrogenic differentiation of human synovium-derived stem cells. Biosci Rep 2017; 37(4). pii: BSR20170347.