

Long non-coding RNA NNT-AS1 promotes cholangiocarcinoma cells proliferation and epithelial-to-mesenchymal transition through down-regulating miR-203

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ABSTRACT

Background: Cholangiocarcinoma (CCA) is a serious malignant tumor. Long non-coding RNA NNT-AS1 (NNT-AS1) takes crucial roles in several tumors. So, we planned to research the roles and underlying mechanism of NNT-AS1 in CCA.

Results: NNT-AS1 overexpression was appeared in CCA tissues and cell lines. Proliferation was promoted by NNT-AS1 overexpression in CCLP1 and TFK1 cells. Besides, NNT-AS1 overexpression reduced E-cadherin level and raised levels of N-cadherin, vimentin, Snail and Slug. However, the opposite trend was occurred by NNT-AS1 knockdown. Further, NNT-AS1 overexpression promoted phosphatidylinositol 3 kinase (PI3K)/AKT and extracellular signal-regulated kinase (ERK)1/2 pathways. MiR-203 was sponged by NNT-AS1 and miR-203 mimic reversed the above promoting effects of NNT-AS1. Additionally, insulin-like growth factor type 1 receptor (IGF1R) and zinc finger E-box binding homeobox 1 (ZEB1) were two potential targets of miR-203.

Conclusion: NNT-AS1 promoted proliferation, EMT and PI3K/AKT and ERK1/2 pathways in CCLP1 and TFK1 cells through down-regulating miR-203.

Methods: CCLP1 and TFK1 cells were co-transfected with pcDNA-NNT-AS1 and miR-203 mimic. Bromodeoxyuridine (BrdU), flow cytometry, quantitative reverse transcription polymerase chain reaction (qRT-PCR) and western blot were employed to detect roles and mechanism of NNT-AS1. Interaction between NNT-AS1 and miR-203 or miR-203 and target genes was examined through luciferase activity experiment.

INTRODUCTION

Cholangiocarcinoma (CCA) is an aggressive tumor coming from biliary epithelial cells, and it is divided into several anatomic subtypes: intrahepatic, hilar and extrahepatic [1]. CCA is the 2nd frequent liver malignancy, and the total incidence of global CCA has risen over the past 40 years [2]. Its infiltration mode is distributed in lymphatic, vascular infiltrating sites and lymph node metastasis [3]. Long-term inflammation, damage and repair of biliary epithelial cells can lead to the occurrence of CCA [4]. There are

many current treatment options, including systemic chemotherapy, targeting radiation and surgery resection [5]. However, there is no satisfactory therapeutic effect. The pathogenesis of CCA is complicated and related to many signal cascades and molecules. For example, the pro-inflammatory factors in CCA can induce the inducible nitric oxide synthase, which acts via suppressing DNA repair proteins and apoptosis proteins [6]. A thorough understanding of CCA mechanism, pathogenic genes and complicated interactions of tumor microenvironments can provide optimal treatment options for patients and improve survival.

Long noncoding RNAs (lncRNAs) are a type of noncoding RNAs (ncRNAs) related to cancer biology with over 200 nucleotides in length [7]. Abnormal lncRNAs expression was observed in many tumors, such as breast cancer [8], bladder cancer [9], *etc.* LncRNA nicotinamide nucleotide transhydrogenase-antisense RNA1 (NNT-AS1), a recently determined lncRNA, was proved to play key roles in various cancers. For example, NNT-AS1 elevated osteosarcoma cells proliferation, survival and mobility through down-regulating microRNA-320a (miR-320a) [10]. Besides, NNT-AS1 is also overexpressed in cervical cancer and its inhibition suppresses cell incursion and proliferation via activating β -catenin/Wnt signaling pathway [11]. These reports indicate the tumor promoting roles of NNT-AS1. However, the roles of NNT-AS1 in CCA development are still unclear.

MiRNAs are small (~ 22 nt) endogenous ncRNAs and involved in cell proliferation, survival, *etc* [12, 13]. It has been reported that lncRNAs could interact with miRNA to regulate tumorigenesis and play key roles in the treatment and diagnosis for patients

[14]. MiR-203 is a miRNA that attracted more attention recently. It is usually decreased and works as a tumor inhibitor in cervical cancer [15], gastric cancer (GC) [16], *etc.* What's more, miR-203 has been proved to work as a valuable prognostic marker and promising treatment target for CCA [17]. Therefore, we want to explore the function of NNT-AS1 and the underlying mechanism between NNT-AS1 and miR-203 to further understand the pathogenesis of CCA.

RESULTS

NNT-AS1 overexpression was observed in CCA samples and cell lines

First, we measured NNT-AS1 expression in CCA tissues and the relative regular tissues. NNT-AS1 expression in CCA tissues was notably higher than that in regular tissues ($P < 0.01$, Figure 1A). Figure 1B revealed that NNT-AS1 levels in SG231 ($P < 0.05$), HuCCT1 ($P < 0.05$), CCLP1 ($P < 0.01$) and TFK1 ($P < 0.01$) cells were notably raised contrasted with H69 cell. Our data revealed that NNT-AS1 was overexpressed in CCA.

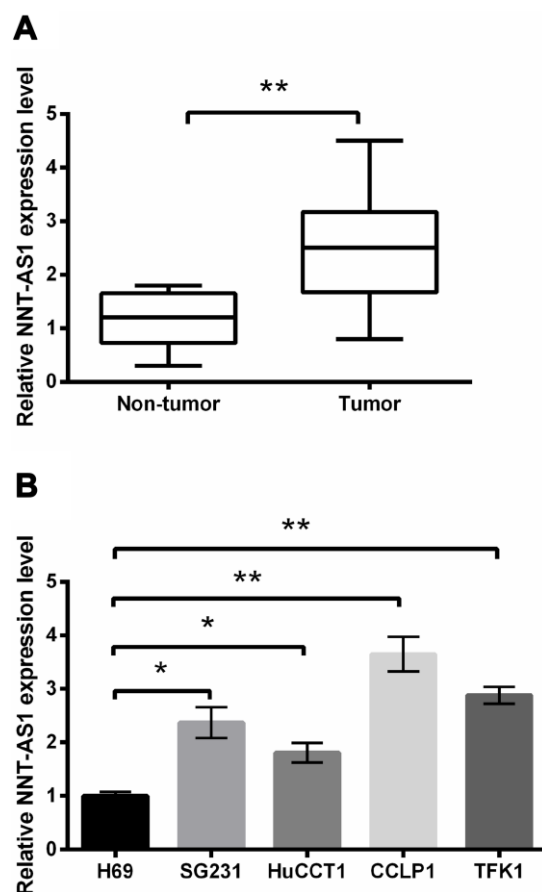


Figure 1. NNT-AS1 overexpression was occurred in CCA. NNT-AS1 levels in CCA tissues (A) and cell lines (B) were measured through qRT-PCR. * $P < 0.05$ and ** $P < 0.01$ contrasted with indicated group.

NNT-AS1 overexpression promoted CCA cell proliferation

Then we attempted to investigate the function of NNT-AS1 in CCLP1 and TFK1 cells through transfection with pcDNA-NNT-AS1 and si-NNT-AS1. Consequents showed that levels of NNT-AS1 were apparently increased in both cells transfected with pcDNA-NNT-AS1 ($P < 0.01$ and $P < 0.001$), while were notably decreased contrasted with relative control in both cells transfected with si-NNT-AS1 (both $P < 0.05$, Figure 2A), suggesting that the NNT-AS1 overexpression and knockdown cell models were successfully established, respectively. Through BrdU experiment, proliferation

was notably raised by NNT-AS1 overexpression (both $P < 0.05$), while was cut down by NNT-AS1 knockdown (both $P < 0.05$, Figure 2B). Similarly, Figure 2C–2D revealed that cyclinD1 level was notably increased after NNT-AS1 overexpression (both $P < 0.01$), while was notably decreased after NNT-AS1 knockdown (both $P < 0.05$). Besides, apoptosis was apparently increased by NNT-AS1 knockdown (both $P < 0.001$, Figure 2E). And similarly, Figure 2F–2G revealed that cleaved-caspase-3 level was obviously increased after NNT-AS1 knockdown ($P < 0.001$). Thus, NNT-AS1 overexpression promoted proliferation, whereas NNT-AS1 knockdown restrained proliferation and caused apoptosis in CCLP1 and TFK1 cells.

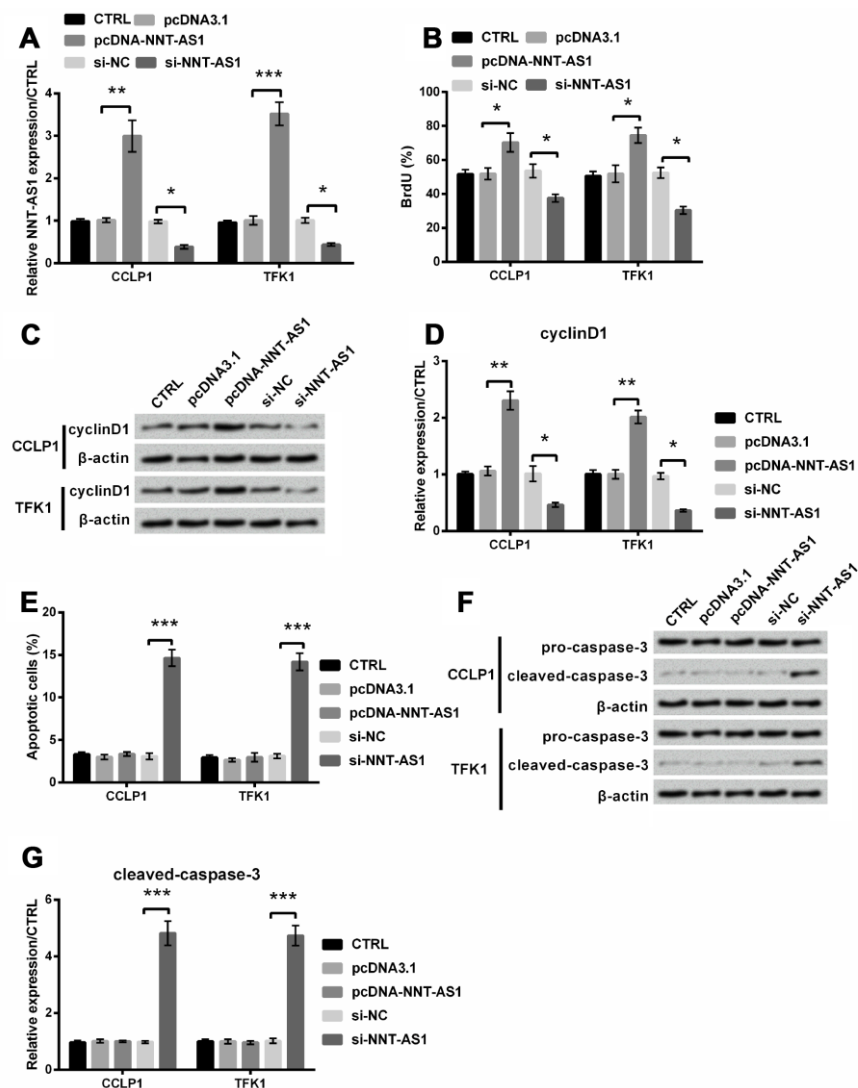


Figure 2. Effects of NNT-AS1 on the growth of CCLP1 and TFK1 cells, which were transfected with pcDNA-NNT-AS1 and si-NNT-AS1. (A) NNT-AS1 level was examined via qRT-PCR. (B) Proliferation was examined via BrdU. Expression of cyclinD1 was examined via western blot (C) and analyzed quantitatively (D) in both cells. (E) Apoptosis was examined via flow cytometry. Expression of cleaved-caspase-3 was examined via western blot (F) and analyzed quantitatively (G) in both cells. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ contrasted with indicated set.

NNT-AS1 overexpression promoted EMT

Next, we examined the effect of NNT-AS1 on EMT. EMT is very important to tumor progression [18]. It accompanies by the loss of expression of E-cadherin, induction of N-cadherin and vimentin [19, 20]. Some transcription elements can suppress E-cadherin expression, such as Snail and Slug, suggesting that these two elements can promote inducing EMT [21]. After 8 h starvation, we used 10 ng/ml TGF β 1 to induce EMT for 12 h. Figure 3A–3D revealed that E-cadherin level was slightly reduced (both $P < 0.05$), while levels of N-cadherin (both $P < 0.05$), vimentin (both $P < 0.01$), Snail (both $P < 0.05$) and Slug (both $P < 0.01$) were notably raised through NNT-AS1 overexpression in

both cells. However, the trends were contrary by NNT-AS1 knockdown. These findings indicated that EMT was promoted by NNT-AS1 overexpression, whereas was suppressed by NNT-AS1 knockdown.

MiR-203 was down-regulated by NNT-AS1

Figure 4A and 4D showed the targeted pairing sequences between NNT-AS1 and miR-203, IGF1R/ZEB1 and miR-203. And the dual luciferase reporter experiment showed that luciferase activities of the vectors carrying NNT-AS1-wt, IGF1R-wt and ZEB1-wt were all notably reduced (all $P < 0.05$, Figure 4B, 4E and 4F). Furthermore, Figure 4C revealed that miR-203 level in cells transfected with pcDNA-NNT-

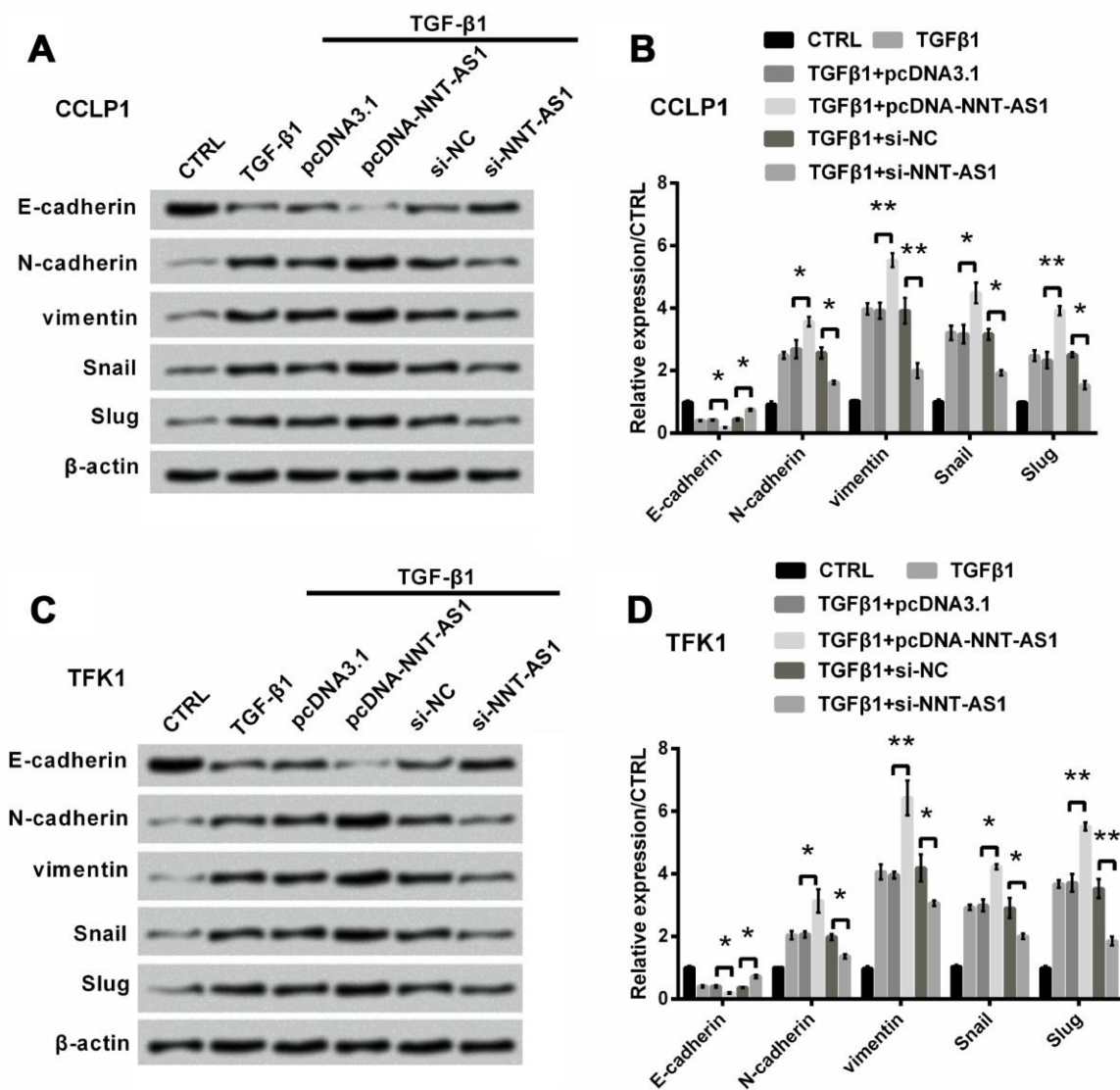


Figure 3. Effect of NNT-AS1 on EMT in CCLP1 and TFK1 cells, which were transfected with pcDNA-NNT-AS1 and si-NNT-AS1. Expression of EMT relative factors was examined via western blot (A, C) and analyzed quantitatively (B, D) in CCLP1 and TFK1 cells. * $P < 0.05$ and ** $P < 0.01$ contrasted with indicated set.

AS1 was notably cut down (both $P < 0.05$), while that in cells transfected with si-NNT-AS1 was notably increased ($P < 0.05$ and $P < 0.01$), suggesting that NNT-AS1 negatively regulated miR-203. Besides, levels of IGF1R and ZEB1 were both decreased by

miR-203 mimic transfection (both $P < 0.05$), while were both notably raised by miR-203 inhibitor transfection (both $P < 0.01$, Figure 4G–4H), indicating that miR-203 down-regulated the expression of IGF1R and ZEB1.

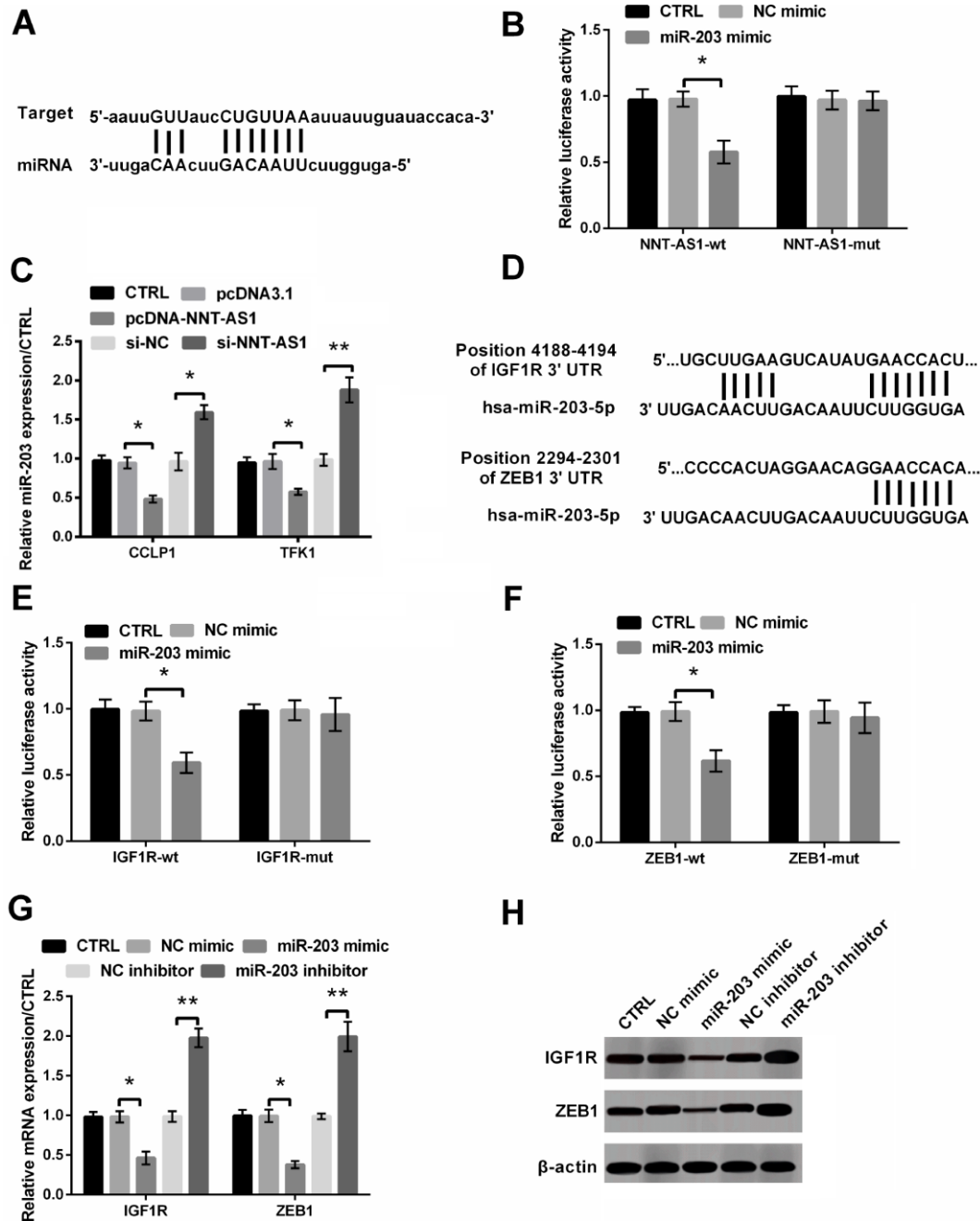


Figure 4. The interaction between NNT-AS1 and miR-203 or miR-203 and IGF1R/ZEB1 was examined. (A) The target matching sequence of NNT-AS1 and miR-203. (B) Luciferase activity was measured after co-transfection with NNT-AS1-wt or NNT-AS1-mut and miR-203 mimic or NC mimic. (C) Level of miR-203 was measured via qRT-PCR when cells were transfected with pcDNA-NNT-AS1 and si-NNT-AS1. (D) The target matching sequence of miR-203 and IGF1R/ZEB1. (E–F) Luciferase activities were measured after co-transfection with IGF1R/ZEB1-wt or IGF1R/ZEB1-mut and miR-203 mimic or NC mimic. (G–H) The mRNA and protein levels of IGF1R and ZEB1 were tested through qRT-PCR and western blot after transfection with miR-203 mimic or miR-203 inhibitor and the relative control. * $P < 0.05$ and ** $P < 0.01$ contrasted with indicated group.

NNT-AS1 overexpression elevated CCA cell proliferation and EMT through down-regulating miR-203

Based on the downward trend of miR-203 expression regulated by NNT-AS1, we assumed that up-regulation of miR-203 might eliminate the tumor promoting effects of NNT-AS1 overexpression on CCLP1 and TFK1 cells. To address this question, miR-203 mimic was transfected into both cells. In Figure 5A, miR-203 mimic notably increased its expression in both cells (both $P < 0.01$). Proliferation was notably raised by NNT-AS1 overexpression (both $P < 0.05$), while was notably decreased (both $P < 0.01$) by the adding of miR-203 mimic in both cells (Figure 5B). Similarly, Figure 5C–5D revealed that the raising level of cyclinD1 was notably decreased by miR-203 mimic ($P < 0.01$ and $P < 0.05$). Besides, for EMT, Figure 5E–5F revealed that level of E-cadherin was slightly reduced by NNT-AS1 overexpression ($P < 0.05$), while was notably raised by miR-203 mimic ($P < 0.05$). Levels of N-cadherin ($P < 0.01$), vimentin ($P < 0.01$), Snail ($P < 0.05$) and Slug ($P < 0.05$) were notably increased by NNT-AS1 overexpression, while were notably decreased ($P < 0.01$, $P < 0.01$, $P < 0.05$ and $P < 0.05$) by the adding of miR-203 mimic in CCLP1 cell. The same trend was appeared in TFK1 cell (Figure 5G–5H). These consequents revealed that NNT-AS1 overexpression elevated proliferation and EMT by down-regulating miR-203.

NNT-AS1 overexpression promoted the activation of PI3K/AKT and ERK1/2 signal pathways through down-regulating miR-203

Finally, Figure 6A–6D revealed that phosphorylation levels of PI3K and AKT were notably raised ($P < 0.01$, $P < 0.01$, $P < 0.001$ and $P < 0.01$) by NNT-AS1 overexpression, while were apparently decreased ($P < 0.01$, $P < 0.05$, $P < 0.01$ and $P < 0.05$) by the adding of miR-203 mimic in CCLP1 and TFK1 cells. And Figure 6E–6H revealed that phosphorylation level of ERK1/2 was obviously raised by NNT-AS1 overexpression (both $P < 0.01$), while was notably cut down through miR-203 mimic (both $P < 0.01$) in CCLP1 and TFK1 cells. These consequents indicated that NNT-AS1 overexpression elevated PI3K/AKT and ERK1/2 pathways' activation through down-regulating miR-203.

NNT-AS1 down-regulated other miRNAs in CCA

In addition to miR-203, the expression of other miRNAs was also reported to be regulated by NNT-AS1, such as miR-363 [21], miR-129-5p [22] and miR-142-3p [23]. Here, we examined whether these miRNAs were regulated by NNT-AS1 in CCA. From Figure 7A–

7B, we found that the expression of miR-363, miR-129-5p and miR-142-3p was all notably decreased by NNT-AS1 overexpression in CCLP1 ($P < 0.05$, $P < 0.05$ and $P < 0.01$) and TFK1 (all $P < 0.05$) cells, while was all dramatically raised by NNT-AS1 knockdown in these cells (all $P < 0.01$). These findings indicated that other miRNAs might also involve in the regulation of NNT-AS1 in CCA.

DISCUSSION

CCA is an uncommon biliary malignant tumor that is prone to lymphatic metastasis and it is the most common primary liver malignancy [24]. Although there are several treatments such as systemic chemotherapy, targeting radiation and surgery resection, most patients are diagnosed as un-removable disease stages, resulting in an overall survival of $< 30\%$ during 5 years [5, 25]. NNT-AS1, a newly identified lncRNA, is researched in its infancy. It has been proved that the overexpression of NNT-AS1 in cervical cancer and hepatocellular carcinoma takes promotion roles in cancer growth [11, 26]. But its role and mechanism in CCA is still unknown. As far as we know, this is the first report to demonstrate the functions and mechanism of NNT-AS1 in CCA. We observed that NNT-AS1 was overexpressed in CCA tissues and cell lines and took promotion roles in CCA growth presented as elevating cell proliferation, EMT and PI3K/AKT and ERK1/2 pathways via down-regulating miR-203 in CCLP1 and TFK1 cells. Our study provided evidence that NNT-AS1 might play promotion roles in CCA development and work as a potential target for CCA treatment.

NNT-AS1, a newly determined lncRNA, was proved to play key functions in tumor development. For instance, Wang et al. observed that NNT-AS1 elevated GC cell proliferation, cell cycle progression and incursion via down-regulating miR-363 [21]. Shen et al. proved that NNT-AS1 was overexpressed in lung cancer and elevated cells growth and incursion through down-regulating miR-129-5p [22]. However, the function of NNT-AS1 in CCA has not been reported. Additionally, EMT, a basic biological process, is involved in many pathology events, such as cancer progression and malignant transformation [27]. Li et al. found that NNT-AS1 knockdown could obstruct breast cancer cells EMT formation [23], suggesting the positive regulatory relation between NNT-AS1 and EMT. Here, we found that NNT-AS1 was also highly expressed in CCA tissues and cell lines. What's more, NNT-AS1 increased proliferation, elevated EMT process and the activation of PI3K/AKT and ERK1/2 pathways in CCLP1 and TFK1 cells. These findings proposed a potential goal for monitoring and treating CCA.

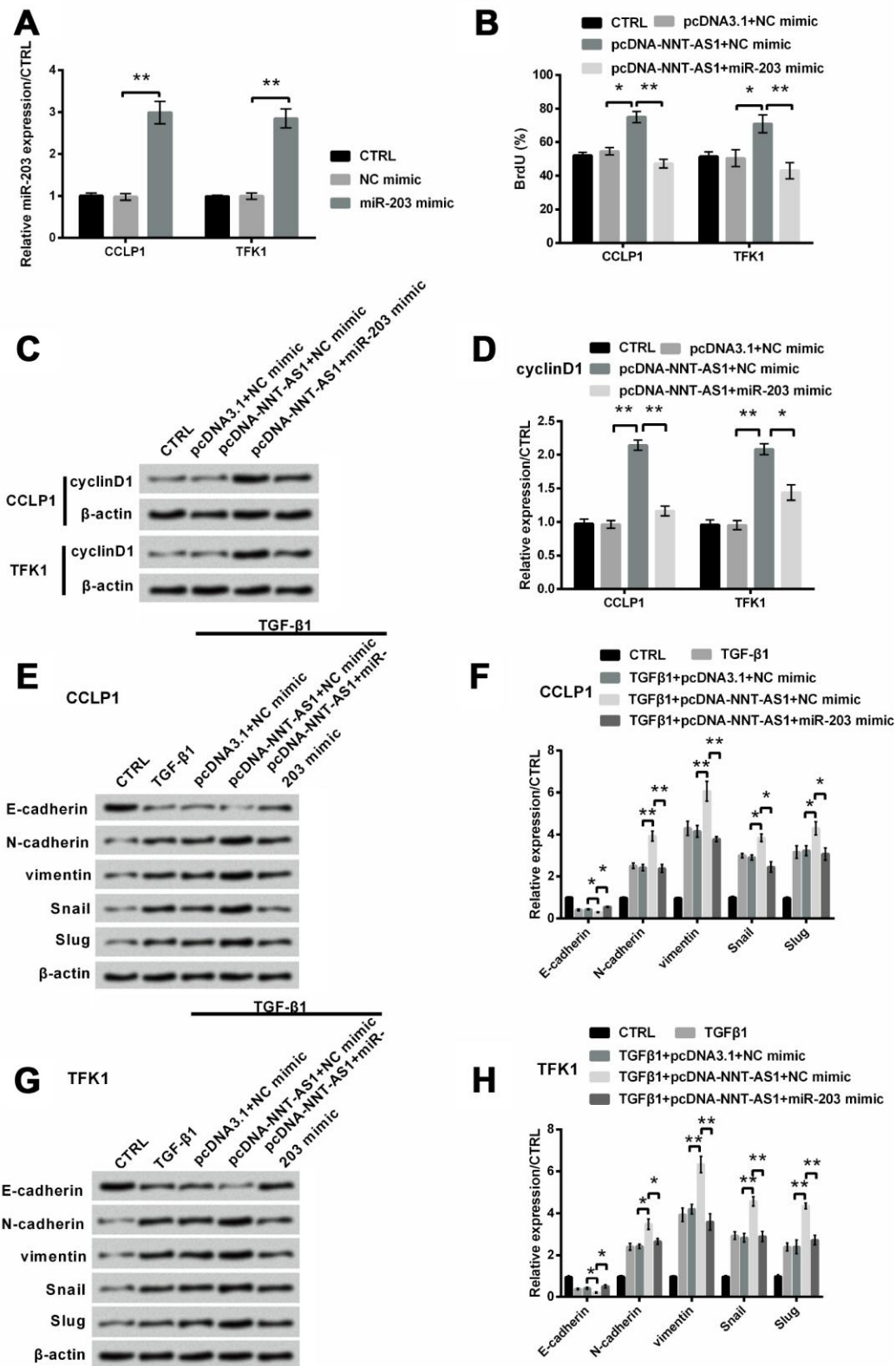


Figure 5. Mechanism of NNT-AS1 on proliferation and EMT in CCLP1 and TFK1 cells after co-transfection with pcDNA-NNT-AS1 and miR-203 mimic. (A) Standard of miR-203 was examined via qRT-PCR by transfection with miR-203 mimic. (B) Proliferation was examined via BrdU. Expression of cyclinD1 was examined via western blot (C) and analyzed quantitatively (D) in both cells. Expression of EMT related factors was inspected via western blot (E, G) and analyzed quantitatively (F, H) in CCLP1 and TFK1 cells. * $P < 0.05$ and ** $P < 0.01$ contrasted with indicated set.

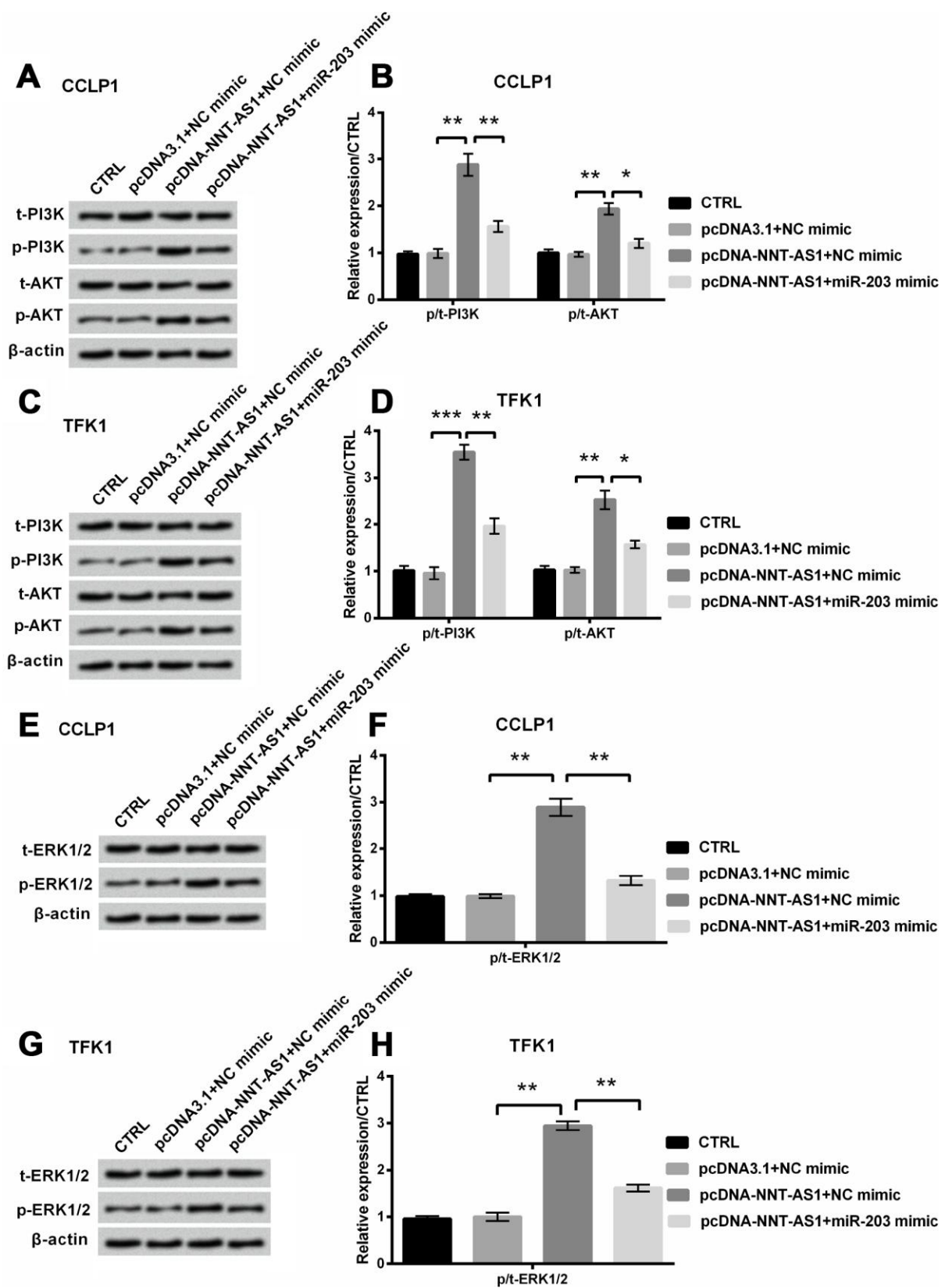


Figure 6. Mechanism of NNT-AS1 on PI3K/AKT and ERK1/2 passageways in CCLP1 and TFK1 cells, which were co-transfected with pcDNA-NNT-AS1 and miR-203 mimic. Expression of PI3K and AKT was measured via western blot (A, C) and analyzed quantitatively (B, D) in both cells. Expression of ERK1/2 was examined via western blot (E, G) and analyzed quantitatively (F, H) in both cells. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ contrasted with indicated set.

Increasing evidence shows the key roles of miRNAs in tumor growth, progression and metastasis [28]. MiR-203, an attractive miRNA, was proved to take a critical role in regulating cell growth and migration [29]. Chi et al. found that miR-203 restrained cell growth, incursion and migration in lung cancer [30]. However, Liu et al. reported that miR-203 elevated cell growth, differentiation and migration in osteoblasts. These researches indicated that miR-203 could play pro-tumor or anti-tumor roles in tumor development, which might rely on the specific cell situation. Besides, many researches revealed that lncRNAs could interact with miRNAs as miRNA sponges to regulate its roles in tumor development [21], NNT-AS1 is no exception. For instance, NNT-AS1 elevated proliferation and incursion of lung cancer cells through regulating down-miR-129-5p

[22]. Besides, NNT-AS1 promoted breast cancer cells growth through down-regulating miR-142-3p [23]. For the first time, we found the regulatory mechanism between NNT-AS1 and miR-203 in CCA. NNT-AS1 could sponge miR-203 and down-regulate the expression of miR-203. And the adding of miR-203 mimic could reverse the promoting effects of NNT-AS1 on proliferation, EMT and PI3K/AKT and ERK1/2 pathways in CCLP1 and TFK1 cells, these findings were consistent with Li et al.'s study that miR-203's low expression was occurred in CCA [17]. And we also found that NNT-AS1 could regulate the expression of miR-363, miR-129-5p and miR-142-3p in CCLP1 and TFK1 cells, suggesting that miRNAs other than miR-203 might also be participated in the regulation of NNT-AS1 in CCA. Additionally, it has been reported that ZEB1, a key factor in controlling

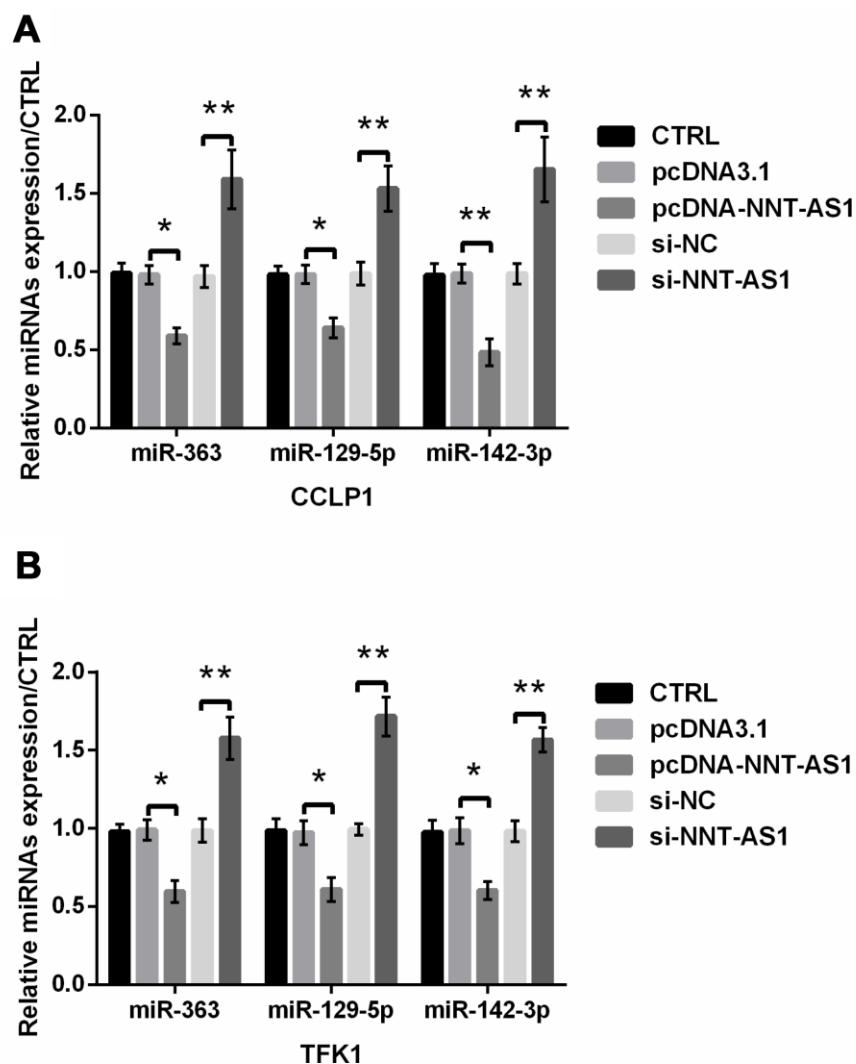


Figure 7. NNT-AS1 regulated other miRNAs in CCA. The expression of miR-363, miR-129-5p and miR-142-3p was examined via qRT-PCR in CCLP1 (A) and TFK1 (B) cells. * $P < 0.05$ and ** $P < 0.01$ contrasted with indicated set.

EMT [31], could be directly targeted by miR-203 in gastric cancer cells [39]. And IGF1R is a polypeptide trophic factor, which is mainly mediated by PI3K/AKT pathway [40]. Here, we found that ZEB1 and IGF1R were two potential candidate targets of miR-203 in CCA cells. Further work is required to explore whether the regulatory mechanism between miR-203 and ZEB1/IGF1R is involved in the cancer promoting effect of NNT-AS1 to enrich the regulatory theoretical basis of NNT-AS1.

PI3K/AKT, a classical pro-survival signal transduction passageway, regulates lots of biological processes, such as proliferation and apoptosis [32]. Downstream effectors of AKT are related to growth, survival and metabolic relative pathways [33], such as vimentin, which is a key member of EMT. PI3K/AKT pathway was proved to be involved in regulating CCA [34]. For example, Wang et al. found that lncRNA MALAT1 elevated CCA cell growth and incursion through promoting the activation of PI3K/AKT pathway [35]. ERK, as a Mitogen-activated protein kinase (MAPK) pathway, is proved to regulate various cellular roles, such as proliferation, incursion and migration to response to stimulation [36]. Menakongka et al. found that the promotion of PI3K and ERK1/2 played positive roles in the invasion of CCA [37]. Besides, several studies have reported the relation between miR-203 and these pathways. For example, miR-203 plays cardio-protective roles by inactivating PI3K/AKT pathway [38]. MiR-203 inhibition elevates GC cell incursion and motility through increasing phosphorylation level of ERK1/2 and Slug expression and the decreased E-cadherin expression [16], suggesting that down-regulation of miR-203 takes pro-tumor roles through activating ERK1/2 and EMT. Consistently, we found that the adding of miR-203 mimic could reverse the promoting effects of NNT-AS1 on EMT and the activation of PI3K/AKT and ERK1/2 pathways in CCLP1 and TFK1 cells, indicating the inhibitory role of miR-203 in EMT and these two pathways. Furthermore, we firstly built up the regulatory mechanism among NNT-AS1, miR-203 and PI3K/AKT and ERK1/2 passageways in CCA that NNT-AS1 promoted CCA cells proliferation, EMT and these pathways' activation via down-regulating miR-203. Our findings further qualify the functional mechanism of NNT-AS1 in CCA.

CONCLUSION

NNT-AS1 overexpression was occurred in CCA and promoted proliferation, EMT and PI3K/AKT and ERK1/2 pathways via down-regulating miR-203 in CCLP1 and TFK1 cells. Our data revealed that

NNT-AS1 worked as an oncogene in CCA growth through down-regulating miR-203, offering a latent monitoring and therapeutic method for CCA.

MATERIALS AND METHODS

Clinical specimens

CAC tissues and normal bile duct tissues samples (n = 20) were got from The First Affiliated Hospital of Zhengzhou University (Zhengzhou China). The First Affiliated Hospital of Zhengzhou University ethics committee agreed with the present study and the written informed consent was got from all patients. The definite pathological diagnosis was built up in patients who underwent surgery but had not got chemotherapy or radiotherapy between January 2014 and December 2015.

Cell

Human CCA cell lines SG231, CCLP1, HuCCT1, TFK1 and 1 noncancerous cholangiocyte cell line (H69) were got from Japanese Cancer Research Resources Bank (Ibaraki City, Japan). SG231 cells were kept in OPTI-MEM medium (Invitrogen, Carlsbad, CA, USA) with 5% fetal bovine serum (FBS, HyClone, Logan, UT, USA). CCLP1 cells were kept in DMEM medium (Sigma, St. Louis, MO, USA) with 10% FBS (HyClone). Besides, TFK1 and HuCCT1 cells were kept in RPMI 1640 medium (Invitrogen) with 10% FBS (HyClone). H69 cells were kept in BEBM Basal Medium (Lonza, Basel, Switzerland) adding with growth elements (BEGM SingleQuot Kit) with 10% FBS (HyClone). All cells were kept in a wet 37°C environment with 5% CO₂. Experiments were conducted when cells reached 80% confluence and were performed in "serum-free medium" (SFM).

qRT-PCR

RNA was gathered via Trizol reagent (Invitrogen). For NNT-AS1, First Strand cDNA Synthesis Kit (Invitrogen) was employed to do reverse transcription. The mRNA expression of NNT-AS1 was examined through the standard SYBR-Green RT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China) with the GAPDH as inside comparison. For miRNAs, Taqman MicroRNA Reverse Transcription Kit to get cDNA and Taqman Universal Master Mix II with the TaqMan MicroRNA Assay (all from Applied Biosystems, Foster City, CA, USA) to do qPCR with U6 as internal comparison.

Proliferation experiment

A commercially available kit (Roche, Basel, Switzerland) was used to examine proliferation with

BrdU incorporation. 1 mg/ml BrdU was employed to keep with cells for 6 h. A fluorescein isothiocyanate (FITC)-labeled antibody (Abcam, Cambridge, MA, USA) was employed to incubate label cells against BrdU for 30 min. Data was got from a fluorescence microplate reader (Millipore, Boston, MA, USA).

Apoptosis experiment

PI and FITC-conjugated Annexin V staining (BD Pharmingen, San Diego, CA, USA) was employed in this part. 1×10^6 /ml cells were gathered and centrifuged at $111.8 \times g$ for 5 min. The remaining cells were washed through PBS, then were centrifuged and resuspended via $1 \times$ Binding Buffer and kept in darkness for 10-15 min. Apoptosis was examined via flow cytometry (Beckman Coulter, Fullerton, CA, USA).

Western blot

Protein was got through RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) with protease inhibitors (Roche) and was quantified via BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). After isolating protein samples through SDS-PAGE, the gel was transferred to PVDF membrane, which was fully blocked through a protein-free blocking solution (Sangon Biotech, Shanghai, China). Primary antibodies specific against cyclinD1 (ab16663, Abcam), pro-caspase-3 (ab183179), β -actin (ab8226), cleaved-caspase-3 (ab49822), E-cadherin (ab15148), N-cadherin (ab95440), vimentin (ab137321), Snail (ab53519), Slug (ab27568), p-phosphatidylinositol 3 kinase (PI3K) (ab182651), t-PI3K (ab140307), t-AKT protein kinase B (AKT, ab179463), p-AKT (ab38499), t-extracellular signal-regulated kinase (ERK, ab54230) and p-ERK (ab214362) were kept with membrane for 10 min and then put in 4°C in the night. Membrane was kept with secondary antibodies in the room for 1 h after cleaning. Add 200 μ l Immobilon Western Chemiluminescent HRP Substrate (Millipore) to mantle its surface after washing for 6 times. Bands were got through Image Lab™ Software (Bio-Rad).

Transfection

Full-length of NNT-AS1 sequences was constructed into pcDNA3.1 plasmid, referring to pcDNA-NNT-AS1. Si-NNT-AS1, si-NC, miR-203 mimic and negative control (NC) were synthesized from GenePharma Co., Ltd (Shanghai, China). Transfections were done through Lipofectamine 2000 (Invitrogen) until cells were reached 50% confluence. Transfection efficiency was examined through qRT-PCR. 48 h was the harvest moment in the latter experiments.

Dual luciferase activity experiment

The online DIANA tools were employed to obtain a potential binding site of NNT-AS1 and miR-203. Cells (3×10^4 cells/well) were kept in 24-well plates. To examine the targeting relationship between NNT-AS1 and miR-203, the NNT-AS1 sequences including the assumed binding site with miR-203 were cloned into a pmirGLO Dual-luciferase miRNA target expression vector (Sangon Biotech). Cells were co-transfected with pmirGLO-NNT-AS1-wild type (wt)/mutated type (mut) reporter plasmids and miR-203 mimic through Lipofectamine 2000 (Invitrogen). Additionally, according the previous studies [39–41] and the target gene prediction website (http://www.targetscan.org/vert_72), we found the potential targets of miR-203 in PI3K/AKT pathway and EMT, which were insulin-like growth factor type 1 receptor (IGF1R) and zinc finger E-box binding homeobox 1 (ZEB1), respectively. The 3'-UTR sequences of IGF1R/ZEB1 including assumed binding site with miR-203 were cloned into luciferase-expressing pGL3-Promoter vectors (Promega, Madison, WI, USA). Cells were co-transfected with IGF1R/ZEB1-wt/mut and miR-203 mimic via Lipofectamine 2000 (Invitrogen). Relative luciferase activity was examined via Dual-Luciferase Reporter Assay System (Promega).

Statistics

Each experiment was iterated for 3 times. Results were displayed as mean \pm SD. Graphpad 6.0 statistical software (GraphPad Software Inc., La Jolla, CA, USA) was employed to do analysis. *P*-values were computed through a one-way analysis of variance (ANOVA) or *t*-test. *P*-value < 0.05 was a notable consequent.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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