MicroRNA-1307-3p accelerates the progression of colorectal cancer via regulation of TUSC5

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Abstract. The aim of the present study was to explore the roles of microRNA-1307-3p (miR-1307-3p) in colorectal cancer (CRC). Firstly, the expression level of miR-1307-3p in CRC cells was measured using reverse transcription-quantitative PCR. Subsequently, Cell Counting Kit-8 and Transwell invasion assays were performed to evaluate the effects of miR-1307-3p on CRC cell proliferation and invasion, respectively. Bioinformatics tools and dual luciferase reporter assays were used to validate the targets of miR-1307-3p. Rescue experiments were performed to confirm tumor suppressor candidate 5 (TUSC5) as a functional target of miR-1307-3p. miR-1307-3p levels were revealed to be upregulated in CRC cells when compared with the normal human epithelial cell line. Knockdown of miR-1307-3p inhibited CRC cell growth and invasiveness. Bioinformatics analysis and dual-luciferase activity reporter assays demonstrated that miR-1307-3p binds the 3'-untranslated region of TUSC5. Finally, rescue experiments validated that miR-1307-3p was able to regulate CRC cell behaviors via regulating TUSC5 expression. Together, the current results indicate that miR-1307-3p functions as an oncogenic miRNA via targeting TUSC5 in CRC.

Introduction

There are ~376,300 new colorectal cancer (CRC) cases according to the data from the Chinese National Cancer Center in China in 2015 (1,2). The treatment strategies for CRC include surgery, chemotherapy and radiation therapy. However, the 5-year overall survival rate for CRC patients at early stages is as high as 90%, with an extremely low rate 13% for patients at stage IV (3).

MicroRNAs (miRNAs/miRs) are RNAs with a length of 19-22 nucleotides, which lack the ability to code for proteins (4). They primarily bind the 3'-untranslated regions (UTRs) of target genes to repress gene expression (4). miRNAs have been demonstrated to be aberrantly expressed in CRC, and they have the potential to be developed as diagnostic or treatment biomarkers. miR-17 has an elevated expression in both CRC tissues and cell lines (5). The overexpression of miR-17 promotes CRC cell metastasis, while knockdown of miR-17 causes the opposite effects via targeting salt inducible kinase 1, indicating the oncogenic role of miR-17 (5). Furthermore, the expression levels of miR-143-3p have been reported to decrease in CRC tissues, and functional analyses revealed that miR-143-3p was able to suppress CRC cell proliferation, migration and invasion capacities via regulating catenin-δ1 (6). Moreover, abnormal expression levels of miR-1307-3p were identified in human cancers, including breast cancer and hepatocellular carcinoma (7,8). In breast cancer, miR-1307-3p was revealed to be upregulated in cancer tissues and increased miR-1307-3p expression increased the risk of death (7). Functional analyses revealed that miR-1307-3p overexpression stimulates breast cancer cell growth through targeting SET and MYND domain-containing 4 (SMYD4) (7). In addition, miR-1307-3p was revealed to be upregulated in hepatocellular carcinoma tissues at advanced tumor stages, in large tumors and was associated with a less favorable overall clinical outcome (8). *In vitro* and *in vivo* analyses indicated that the knockdown of miR-1307-3p inhibited tumor progression via regulating Disabled Homolog 2 interacting protein (8). To date, it is unclear whether miR-1307-3p has a role in the development of CRC.

Tumor suppressor candidate 5 (TUSC5) is a protein that is widely expressed in brown adipocytes and peripheral neurons (9,10). In recent years, the roles of TUSC5 in various tumor types have been gradually recognized. For example, TUSC5 expression was reported to be regulated by miR-3188, affecting breast cancer cell proliferation, migration and apoptosis capacity (11). In addition, TUSC5 was also revealed to be downregulated, and served as target for miR-484, to influence the malignant phenotypes of hepatocellular carcinoma cells (12).

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In the present study, the expression levels of miR-1307-3p were explored in CRC cells. Additionally, functional assays were performed to investigate the functions of miR-1307-3p in CRC progression. Furthermroe, the mechanisms of action that mediated the functions of miR-1307-3p were explored.

Materials and methods

Cell lines and transfection. The normal human epithelial cell line NCM460 and CRC cells, including SW480 and SW620, were obtained from the American Type Culture Collection were grown in DMEM (cat. no. A4192101; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (cat. no. 16000044; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (cat. no. 15140122; Gibco; Thermo Fisher Scientific, Inc.) in a 37˚C humidified atmosphere containing 95% atmospheric air and 5% CO_2. The miR-1307-3p inhibitor (5'-CACGACCGACGC CACGCCGAGU-3') and control miRNA (miR-con, 5'-AGG CCAGCCACGGCGCAUCCAC-3') were synthesized by Guangzhou RiboBio Co., Ltd. Specific small interfering (si)RNA targeting TUSC5 (si-TUSC5; 5'-GGAGAACAA GGAUGACCAATT-3') and the corresponding control sequence (siR-con; 5'-CAGTCGCGTTTGCGACTGGTT-3') were also purchased from Guangzhou RiboBio Co., Ltd. Transfections were conducted using Lipofectamine® 2000 (cat. no. 16168019; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions (final concentrations: miRNA, 200 nM; siRNA, 100 nM). Cells (SW480 and SW620) were transfected for 48 h before being collected for subsequent analyses.

Microarray analysis. The GSE123040 dataset (13) was downloaded from the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/gds/?term=gse123040) and was used to determine the expression levels of miR-1307-3p in CRC tissues and normal tissues.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Cultured cells (SW480 and SW620) with or without oligonucleotides transfection, were lysed to isolate RNA using TRIzol® reagent (cat. no. 15596018; Thermo Fisher Scientific, Inc.) according to the manufacturer's instruction. For miRNA, complementary DNA (cDNA) was synthesized using the miScript II RT kit (cat. no. 4366596; Thermo Fisher Scientific, Inc.). For mRNA, cDNA was synthesized using the PrimeScript RT Reagent kit (cat. no. 6210A, Takara Biotechnology, Inc.). Quantitative PCR was conducted using an ABI 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using SYBR Green mix obtained from Takara Biotechnology Co., Inc. (cat. no. RR820A). Primers sequences used in the present study were as follows: miR-1307-3p forward, 5'-TGCGGGTCCAGTTTTCCCAGG AA-3' and reverse, 5'-CCAGTGCAGGGTCCGAGGT-3'; U6 snRNA forward, 5'-TGCGGGTGCTCGCTTCGCAGC-3' and reverse, 5'-CCAGTGCAGGGTCCGAGGT-3'; TUSC5 forward, 5'-ATTAGTAAAGTTGTTT-3' and reverse, 5'-CAA AAAACTCTAAAAAAA-3'; GAPDH forward, 5'-AACGTG TCAGTOGTGGACCTG-3' and reverse, 5'-AGTGGGTGT CGCTGTFGAAGT-3'. The procedure used was as follows: 1 cycle at 94˚C for 10 min, 40 cycles at 95˚C for 10 sec, 56˚C for 30 sec and 70˚C for 30 sec. U6 snRNA or GAPDH was regarded as internal control gene for miR-1307-3p or TUSC5, respectively. Relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (14).

Cell proliferation assay. Cells (SW480 and SW620) with or without oligonucleotides transfection, were plated into a 96-well plate at a density of $3x10^3$ cells/well and then the cell proliferation rate was detected using the cell counting kit-8 (cat. no. C0037; CCK-8, Beyotime Institute of Biotechnology) assay according to the supplier's instruction. At the indicated times, 10 μl CCK-8 was added to the well and further incubated for 2 h at 37˚C. Finally, optical densities for each well were measured at 450 nm.

Cell invasion assay. Matrigel® (cat. no. 40480; BD Biosciences) was used to pre-coated 8-μm chamber (cat. no. 354480, Corning, Inc.) at room temperature for 24 h. Subsequently, $1x10^5$ cells suspended in serum-free DMEM were plated in the upper chamber, while the DMEM containing 10% FBS (cat. no. 16000044; Gibco; Thermo Fisher Scientific, Inc.) was plated in the lower chamber. After 48 h incubation, invading cells were fixed with 90% methanol at room temperature for 30 min, stained with 0.1% crystal violet at room temperature for 15 min, and counted under inverted light microscope (magnification, x200).

Bioinformatics analysis. TargetScan 7.2 (http://www.targetscan.org/) was utilized to analyze potential targets for miR-1307-3p.

Dual-luciferase reporter assays. TUSC5 3'-UTR containing binding sites for miR-1307-3p were inserted into the pmirGLO plasmid obtained from Promega Corporation to generate wild-type TUSC5 (TUSC5-wt). Mutant TUSC5 (TUSC5-mt) was constructed using the Site-Directed Mutagenesis kit (Takara Bio, Inc.). TUSC5-wt or TUSC5-mt were co-transfected with 100 nM synthetic miRNA into $2x10^3$ CRC cells using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. After transfection for 48 h, the dual-luciferase system (cat. no. E1910, Promega Corporation) was used to detect relative luciferase activities with *Renilla* luciferase activity as internal control according to the manufacturer's instructions.

Statistical analysis. SPSS version 18.0 (SPSS, Inc.) was used to analyze data obtained from three independent experiments. Data are presented as the mean ± SD. Differences in groups were analyzed using the paired Student's t-test (2 groups) or one-way ANOVAs with Tukey's post-hoc test (≥3 groups). $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-1307-3p is upregulated in CRC tissues and cell lines. Firstly, miR-1307-3p expression levels were measured in the CRC and normal cell lines using RT-qPCR. It was revealed that miR-1307-3p expression was significantly elevated in CRC

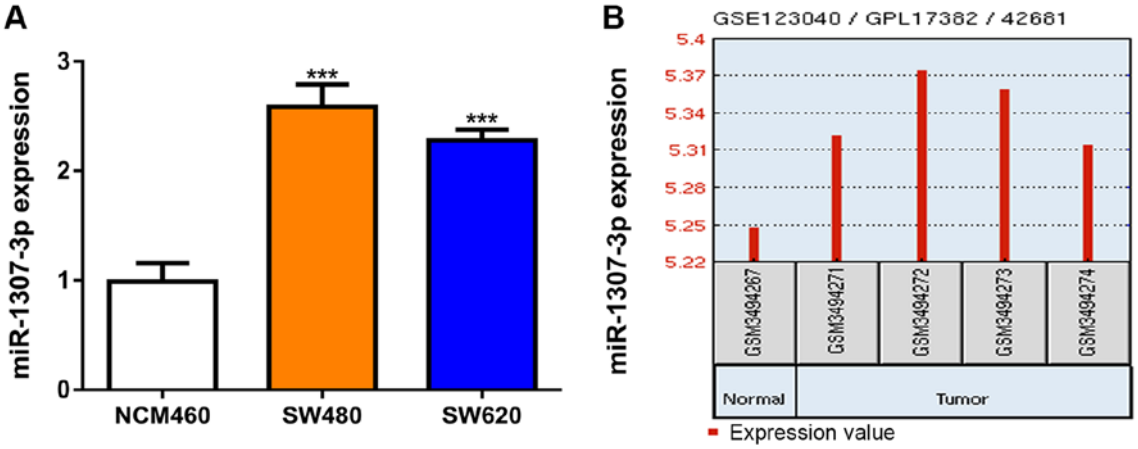

Figure 1. miR-1307-3p is upregulated, while tumor suppressor candidate 5 is downregulated in CRC cells. (A) Expression of miR-1307-3p in CRC cells (SW480 and SW620) and normal cell line (NCM460) was measured using reverse transcription-quantitative PCR. (B) Expression of miR-1307-3p in CRC tissues and normal tissues was obtained from the GSE123040 dataset. ***P<0.001 vs. NCM460. CRC, colorectal cancer; miR, microRNA.

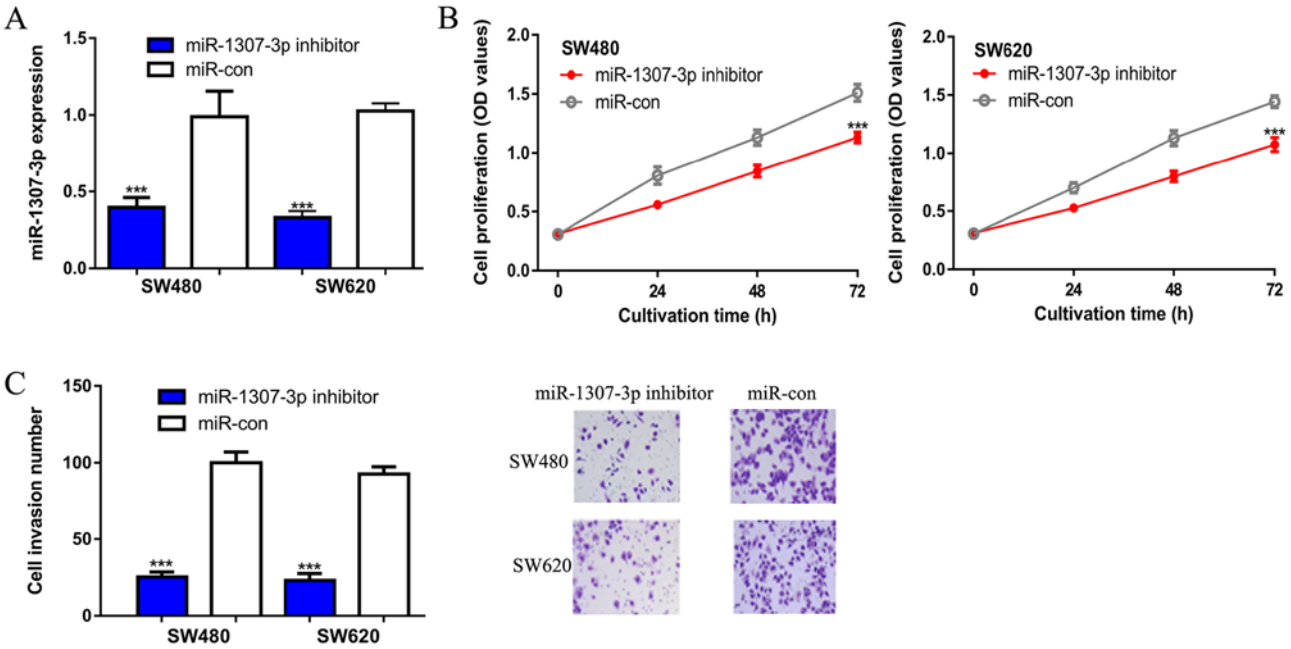

Figure 2. miR-1307-3p regulates CRC cell proliferation and invasion. (A) Expression of miR-1307-3p, (B) cell proliferation and (C) cell invasion (magnification, x200) in CRC cells transfected with miR-1307-3p inhibitor or miR-con. ***P<0.001 vs. miR-con. con, control; CRC, colorectal cancer; miR, microRNA; OD, optical density.

cells compared with the normal cell line (Fig. 1A). Moreover, miR-1307-3p expression levels were increased in CRC tissues compared with normal tissues (Fig. 1B).

miR-1307-3p promotes CRC cell proliferation and invasion. The effects of miR-1307-3p on CRC cell behavior were then investigated via loss-of-function experiments. It was demonstrated that the introduction of a miR-1307-3p inhibitor significantly decreased miR-1307-3p expression levels in CRC cells (Fig. 2A). CCK-8 assays revealed that the optical density value in the miR-1307-3p inhibitor-transfected group was significantly lower compared with the miR-con group, as indicated at 72 h (Fig. 2B). Transwell invasion assays were then performed to evaluate the effect of miR-1307-3p on cell invasion. It was revealed that there were fewer invasive cells in the miR-1307-3p inhibitor transfected group compared with the miR-con group (Fig. 2C). The effect of miR-1307-3p on proliferation was also tested in the normal cell line (NCM460). As indicated in Fig. S1A and B, knockdown of miR-1307-3p repressed NCM460 cell proliferation.

miR-1307-3p targets TUSC5 in CRC. To explore the mechanisms of action behind miR-1307-3p-mediated CRC cell behaviors, the targets of miR-1307-3p were predicted using TargetScan, and it was revealed that TUSC5 represented a potential target (Fig. 3A). Dual-luciferase activity reporter assays revealed that miR-1307-3p inhibitor transfection increased the luciferase activity of CRC cells transfected with TUSC5-wt, but not TUSC5-mt (Fig. 3B). Subsequently, RT-qPCR was performed to analyze the TUSC5 expression

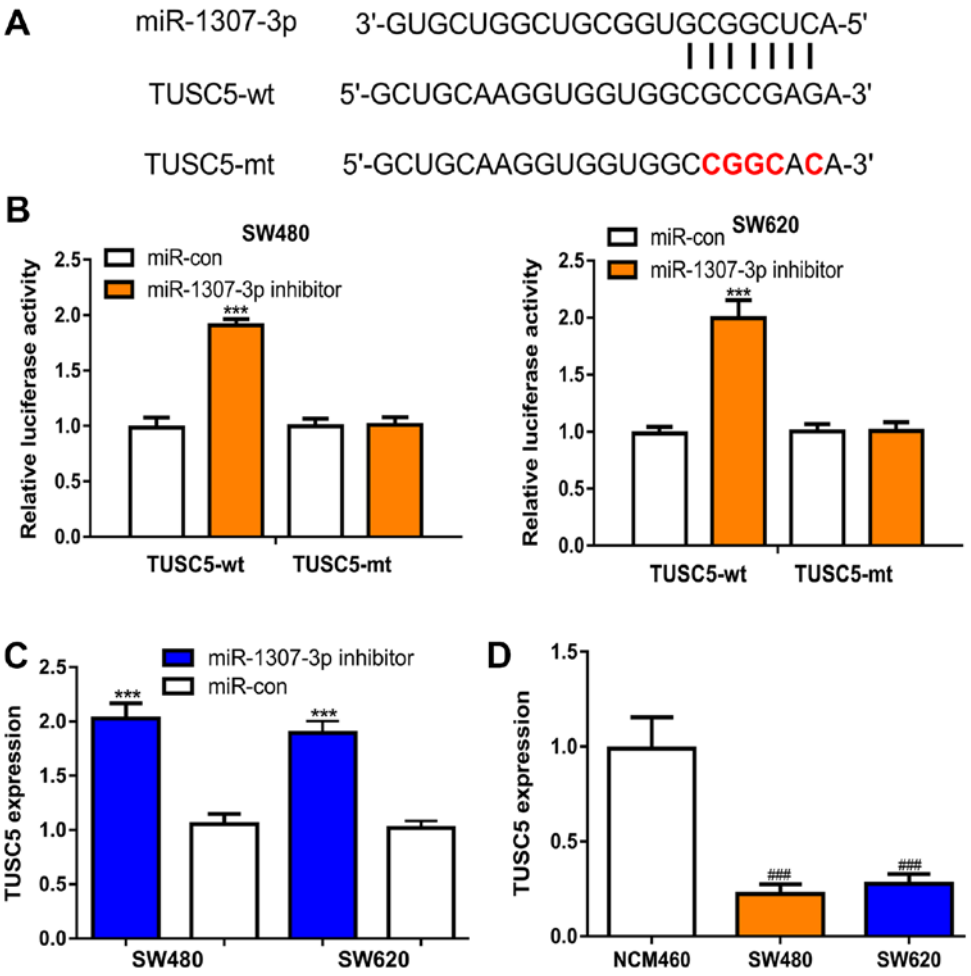

Figure 3. miR-1307-3p directly targets TUSC5 in CRC. (A) The binding region between miR-1307-3p and the TUSC5 3'-untranslated region. (B) Luciferase activity in CRC cells transfected with luciferase vectors or synthetic miRNAs. (C) miR-1307-3p regulated the expression of TUSC5 in CRC cells. (D) Expression of TUSC5 in CRC cells and the normal cell line was measured using reverse transcription-quantitative PCR. ***P<0.001 vs. miR-con; $^{###}$P<0.001 vs. NCM460. con, control; CRC, colorectal cancer; miR/miRNA, microRNA; mt, mutant; TUSC5, tumor suppressor candidate 5; wt, wild type.

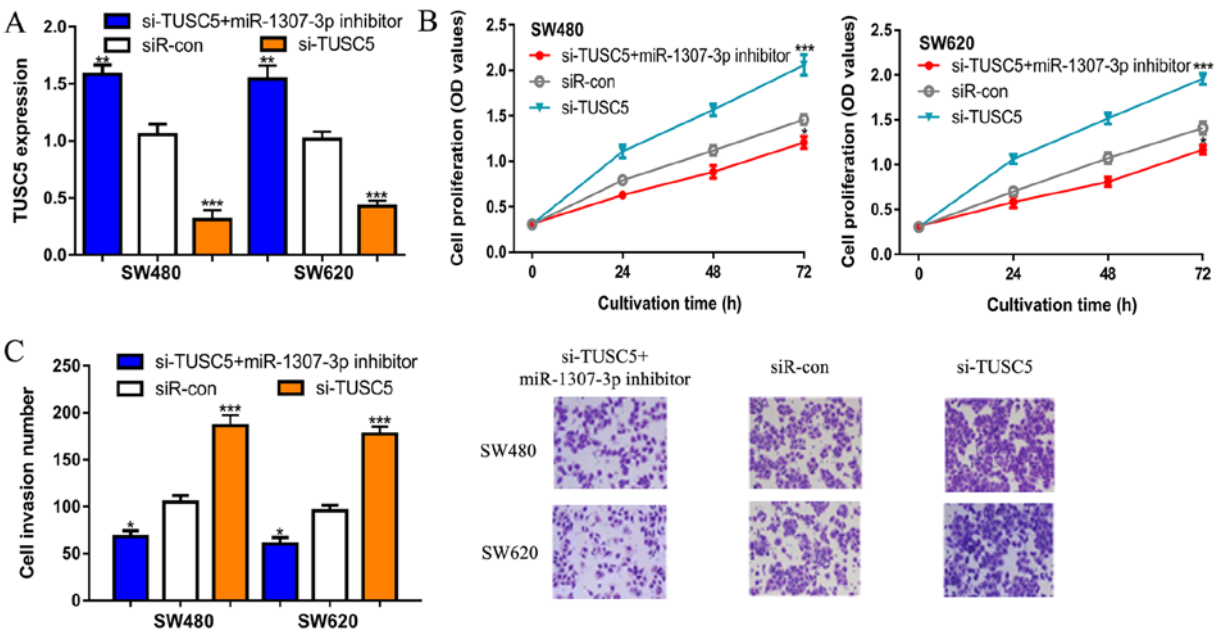

Figure 4. Knockdown of TUSC5 restores the effects of miR-1307-3p on CRC cell behavior. (A) Expression of TUSC5, (B) Cell proliferation and (C) Cell invasion (magnification, x200) in CRC cells transfected with si-TUSC5, siR-con or si-TUSC5 + miR-1307-3p inhibitor. *P<0.05, **P<0.01, ***P< 0.01 vs. siR-con. con, control; CRC, colorectal cancer; siR-con, negative control small interfering RNA; miR, microRNA; OD, optical density; si-TUSC5, small interfering RNA targeting tumor suppressor candidate 5; TUSC5, tumor suppressor candidate 5.

levels in the groups transfected with miR-1307-3p inhibitor or miR-con. TUSC5 expression levels were significantly elevated by the miR-1307-3p inhibitor in comparison with the miR-con (Fig. 3C). Moreover, it was shown that the TUSC5 expression levels were decreased in the CRC cell lines compared with the normal cell line (Fig. 3D).

Knockdown of TUSC5 attenuates the effects of miR-1307-3p on CRC cells. To further confirm the role of TUSC5 in miR-1307-3p-mediated stimulation of CRC cells, rescue experiments were performed. It was demonstrated that the transfection of si-TUSC5 decreased TUSC5 expression levels in CRC cells (Fig. 4A). si-TUSC5 also appeared to partially reverse the effects of miR-1307-3p inhibitor on TUSC5 expression (Figs. 3C and 4A). It was also found that the knockdown of TUSC5 appeared to reduce the effects of miR-1307-3p inhibitor on CRC cell proliferation as indicated at 72 h (Figs. 2B and 4B). Similar results were observed in Transwell invasion assays. Although not directly compared, TUSC5 downregulation appeared to decrease the impact of the miR-1307-3p inhibitor on CRC cell invasion capacity (Figs. 2C and 4C).

Discussion

The mechanisms of action underlying CRC progression have been gradually explored in recent years (15). Numerous miRNAs have been identified to be abnormally expressed in CRC and function as either tumor drivers or suppressors (5,6). Hence, it is essential to fully investigate abnormally expressed molecules associated with CRC carcinogenesis to discover novel biomarkers for prognosis or treatment (16).

In this present study, miR-1307-3p expression levels were revealed to be significantly upregulated in CRC tissues and cell lines compared with normal tissues and cell lines, respectively. Suppressing miR-1307-3p in CRC cells inhibited CRC cell proliferation and invasion *in vitro*. As a tumor-driver miRNA, miR-1307-3p expression levels were upregulated in several cancer types, including breast cancer and hepatocellular carcinoma (7,8). Consistent with previous studies, the current work indicated that miR-1307-3p functions as an oncogenic miRNA to promote CRC progression.

Previous studies have indicated that miR-1307-3p influences cancer progression via regulating the expression of SMYD4 and DAB2 interacting protein (7,8). In the present study, it was demonstrated that TUSC5 was a putative target for miR-1307-3p using TargetScan. In this present study, knockdown of TUSC5 promoted CRC cell proliferation and invasion; and may have partially reversed the effects of miR-1307-3p inhibitor on the malignant phenotypes of CRC cells. However, the mechanisms of action underlying miR-1307-3p-mediated regulation of TUSC5 expression, and the associated mechanisms in CRC remain unclear and require further exploration. In addition, it has to be recognized that the primary limitation of the present work is that the miR-1307-3p/TUSC5 axis was not validated in a xenograft tumor model. In the future work, to address this issue, CRC cells could be inoculated in nude mice and then the synthesized miRNAs injected into the developing tumors at varying time points. Subsequently, the tumor volume and tumor weight as well as markers of metastasis, including ki-67, N-Cadherin and vimentin should be examined to investigate the effects of miR-654-5p on tumor growth and metastasis *in vivo*.

In conclusion, miR-1307-3p was upregulated in CRC cells and regulated CRC cell proliferation and invasion through regulating TUSC5. The present study provided evidence that miR-1307-3p may be therapeutic target for the treatment of CRC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

NY, MY, RZ and MW performed the experiments, analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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