MIR-185-5P AFFECTS THE GROWTH AND METASTASIS OF COLORECTAL CANCER BY NEGATIVELY REGULATING TNNT1

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ABSTRACT

Objective*: The purpose of this study is to elucidate the molecular mechanism of the occurrence and development of colorectal cancer (CRC), and to provide a theoretical basis for possible treatment options for CRC patients.*

Methods: *The expression level of TNNT1 in CRC patients was analysed using the UALCAN database, and the survival curve of CRC patients separated by TNNT1 expression level was analysed using the GEPIA database. MiR-185-5p- and TNNT1-overexpressing CRC cell models were constructed by transfecting cells with miR-185-5p mimics and TNNT1 plasmid. The transcription levels of TNNT1 and miR-185-5p in each group of cells were detected by RT-qPCR. The expression of TNNT1 in the cells was detected using Western blot. The possible binding sequence of miR-185-5p to TNNT1 was predicted using the Targetscan database, and the regulatory relationship between miR-185-5p and TNNT1 was further verified using a luciferase reporter gene assay. Cell Count Kit-8 (CCK-8) and BrdU immunofluorescence staining were used to detect cell proliferation in each group, and the migration and invasion levels of each group were detected by Transwell assay.*

Results: *The expression of TNNT1 was significantly upregulated in CRC patients, and the survival curve results showed that high expression of TNNT1 was significantly correlated with decrease in the survival time of patients. At the same time, RT-qPCR results showed that the transcription level of TNNT1 was significantly increased in CRC cell lines, while the expression of miR-185-5p was significantly reduced. The overexpression of miR-185-5p significantly inhibited the expression of TNNT1 in SW480 cells and functionally inhibited the proliferation and invasion ability of SW480 cells. Double luciferase reporter gene tests and Western blotting results showed that miR-185-5p could directly act on and negatively regulate the expression of TNNT1. In addition, forced overexpression of TNNT1 completely reversed the inhibitory effect of miR-185-5p on SW480 cells.*

Conclusion: *MiR-185-5p inhibits the proliferation and invasion of CRC cells by targeting TNNT1 in CRC, which provides a new therapeutic target for CRC.*

Keywords: *Colorectal cancer, TNNT1, miR-185-5p, growth and metastasis.*

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Introduction

Colorectal cancer (CRC) is one of the most common malignant tumours in the world. CRC causes about 390,000 deaths each year, and the relative survival rate is less than 40%[1, 2]. The diagnosis and treatment technology and efficacy of CRC have made great progress, but the number of CRC patients is still increasing year by year, especially in some developing countries[3]. Therefore, exploring the potential biological mechanisms of the occurrence and development of CRC is of great significance for the clinical prevention and treatment of this type of disease. Troponin T (TNT) is an important protein, with a molecular weight of about 30~35 kDa and containing about 220~300 amino acids, that can regulate the contraction and relaxation of striated muscle[4]. Previous research has shown that TNT may be a biomarker for certain human diseases[5].

For example, assessing the levels of high-sensitivity TNT before and after treatment is important for assessing the risk of cardiac dysfunction during

breast cancer chemotherapy[6]. As one of the subunits of TNT, Troponin T1 (TNNT1) is associated with adrenal myopathy[7]. In addition, TNNT1 has also been associated with breast cancer progression[8]. However, the mechanism of TNNT1 in CRC and its clinical significance have not yet been clarified, and further research is needed.

MicroRNA (miRNA) is a type of single-stranded non-coding RNA containing 18 to 24 nucleotides. More and more studies have shown that certain miRNAs play tumour promoting or tumour suppressive roles in different human tumours[9]. For example, miR-133a-3p plays a tumour suppressive role in HCC[10]. MiR-146b disorders are associated with the occurrence and prognosis of thyroid papillary carcinoma[11]. MiR-26a can regulate the proliferation and metastasis of tumour cells by regulating the PTEN-AKT axis[12]. An important member of miRNA, miR-185-5p is related to apoptosis, epithelial-mesenchymal transition and cytochemical sensitivity of various human cancer cells[13, 14]. Previous studies have shown that lncRNA PDIA3P negatively regulates miR-185-5p in OSCC cells. Silencing PDIA3P through the miR-185-5p/cyclin D2 pathway can inhibit cell proliferation[15]. However, whether miR-185-5p is involved in the regulation of the CRC process and its underlying molecular mechanism is unclear. In this study, biological analysis showed that CRC patients with increased expression of TNNT1 have a worse prognosis. In vitro experiments further showed that TNNT1 is upregulated in CRC tissue cells and has a pro-cancer effect. Further research found that miR-185-5p can negatively regulate TNNT1 and affect the progress of CRC. In summary, this study aimed to clarify the molecular mechanism of the occurrence and development of CRC and to provide a theoretical basis for possible treatment options for CRC patients.

Methods

Cell culture

Human colorectal cancer cell lines HT29, SW480 and HCT116 and normal colon cell line CRL1790 were purchased from the Shanghai Institute of Cell Sciences, Chinese Academy of Sciences. All cells were cultured in DMEM medium (Gibco, USA) with 10% foetal bovine serum (Gibco, USA) and 100 U/ml penicillin/streptomycin (Invitrogen, USA). The cells were placed in a 5% CO_2 incubator at a constant temperature of 37 °C. The culture medium was changed every 2 days, and the cells in log phase were taken for subsequent experiments. MiR-185-5p mimics, inhibitors and the TNNT1 plasmid were purchased from GenePharma (Shanghai, China). Transfection into SW480 cells was accomplished using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions.

BrdU fluorescent staining

Transfected cells were seeded on a cover glass in a 24-well plate and cultured overnight. BrdU (10 μg/mL) was added to the culture medium for 1 hour, and then the cells were fixed in 4% paraformaldehyde for 10 minutes according to the manufacturer's instructions and stained with anti-BrdU antibody (Biocompare, USA).

The coverslips were then counterstained with DAPI to label the nuclei, and images were obtained under a fluorescent microscope.

RT-qPCR

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) and RT-qPCR was performed on the ABI StepOne Plus system using SYBR Green Master Mix (Roche, Switzerland). GAPDH was used as a control for miR-184 and STC2, and its content was calculated by the $2^{-\Delta\Delta ct}$ method. The primers were as follows: miR-185-5p forward primer 5'- GCATGGCTTTGACCACTCTG -3', reverse primer 5'- CTACAGGAGGGTTGGTGTCCCC-3'; TNNT1 forward primer 5'-AACGCGAACGTCAGGCTAAGCT-3', reverse primer 5'-CAGGGAGAAACGACCTGGAG-3'; GAPDH forward primer 5'-AGCCACATCGCTCAGACAC-3', reverse primer 5'-GCCCAATACGACCAAATCC-3'.

Western blotting

Cells were washed with PBS and lysed with a RIAP lysis buffer containing a protease inhibitor (Thermo, USA). Supernatant was collected by high speed centrifugation. The protein was quantified by the BCA method, and the supernatant was heated in a water bath to denature the protein.

The protein was separated by SDS-PAGE and blocked with skimmed milk powder at room temperature for 30 minutes and transferred to a PVDF membrane. After rinsing with TBST, the membrane was added to the culture and incubated with primary antibody at 4 °C overnight. It was then rinsed with TBST solution and incubated with secondary antibody for 1 hour. Thereafter, colour development was performed using an enhanced chemiluminescence kit (Pierce, USA).

Transwell test

An 8 μm pore size Transwell chamber coated with matrix gel was used (not for migration experiments). SW480 cells were trypsinised, centrifuged and resuspended in FBS-free medium. Then, $5x10^4$ cells were placed in the upper chamber and 10% FBS medium was placed in the lower chamber. After 24 h of incubation at 37 °C, cells that failed to migrate or invade were removed from the upper chamber. The Transwell membrane was fixed with 4% paraformaldehyde for 10 min and stained with 0.5% crystal violet. After rinsing the cells with tap water, the migration or invasion of the cells was counted under an inverted microscope.

CCK-8 test

The Cell Count Kit-8 (CCK-8) measurement was performed according to the CCK-8 instruction manual. Stably transfected SW480 cells were seeded in 96-well plates ($1x10^3$ cells/well). CCK-8 solution was added to 96-well plates after 1, 2, 3 and 4 days and incubated at 37 °C for another 2 hours. Finally, the absorbance at 450 nm was measured using a microplate reader.

Statistical analysis

All data were expressed as mean ± standard deviation. Statistical analysis was performed using GraphPad Prism 8.0 for analysis and mapping.

Differences between groups were determined by Student p tests and one-way ANOVA tests, followed by a post hoc test. $P<0.05$ was considered statistically significant.

Results

TNNT1 expression was downregulated in CRC tissues and cell lines

The expression level of TNNT1 in clinical CRC patients was analysed using the UALCAN database (http://ualcan.path.uab.edu/index.html) to explore the expression characteristics of TNNT1 in CRC. The results showed that the expression of TNNT1 in CRC was significantly higher than that in normal people (Figure 1A). In addition, the survival curves of CRC patients with respect to TNNT1 expression was obtained through the GEPIA database (http://gepia.pku.cn/index.html), and it was found that the overall survival of CRC patients with high expression of TNNT1 was significantly worse (Figure 1B). To further explore the expression characteristics of TNNT1 in CRC, Western blotting was used to detect the expression level of TNNT1 in different cell lines. The results suggested that compared with the normal cell line CRL1790, the expression of TNNT1 in CRC cell lines HT29, SW480 and HCT116 was significantly increased (Figure 1C).

At the same time, the expression level of miR-185-5p in different cell lines was detected by RT-qPCR and the results showed that compared with normal cell lines, the expression level of miR-185-5p was significantly reduced in CRC cell lines (Figure ID). In summary, these data indicated that TNNT1 was upregulated in CRC tissues, suggesting its carcinogenic role in CRC progression, and that miR-185-5p may also play a role.

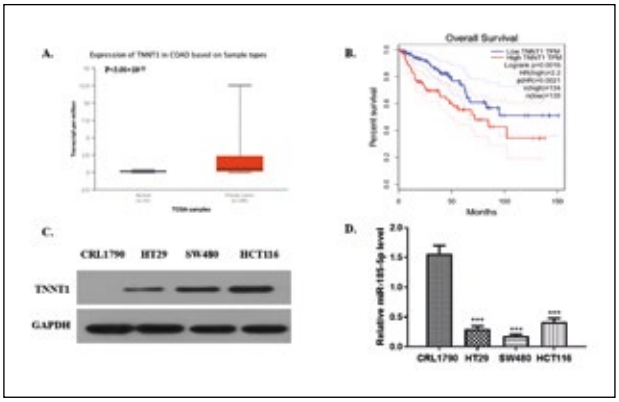

Figure 1: TNNT1 expression was downregulated in CRC tissues and cell lines.

A. Analysis of TNNT1 expression in clinical CRC patients by the UALCAN database. B. Analysis of survival curves in CRC patients according to TNNT1 levels by GEPIA database. C. Analysis of TNNT1 expression in human colorectal cancer cell lines HT29, SW480 and HCT116 and normal colon cell line CRL1790 by Western blotting. D. Analysis of miR-185-5p expression in human colorectal cancer cell lines HT29, SW480 and HCT116 and normal colon cell line CRL1790 by RT-qPCR experiments. ****, p<0.001.*

MiR-185-5p overexpression inhibited CRC cell migration and invasion

To further explore the role of miR-185-5p in CRC, a miR-185-5p-overexpressing SW480 cell model was constructed by transfecting miR-185-5p mimics. In order to investigate the effect of TNNT1 on CRC cells, the CCK-8 test and BrdU immunofluorescence staining were used to detect the proliferation of SW480 cells transfected in each group. CCK-8 experimental results showed that miR-185-5p overexpression promoted SW480 cell proliferation compared to the control group (Figure 2A).

Similarly, the results of immunofluorescence experiments also showed that miR-185-5p overexpression promoted SW480 cell proliferation compared to the control group (Figure 2B). Next, the mi-

gration and invasion ability of CRC cells were tested by Transwell assays and the results showed that miR-185-5p overexpression significantly inhibited the migration and invasion of SW480 cells compared to the control group (Figure 2C and 2D). These results suggested that miR-185-5p may play a role as a tumour suppressor in CRC.

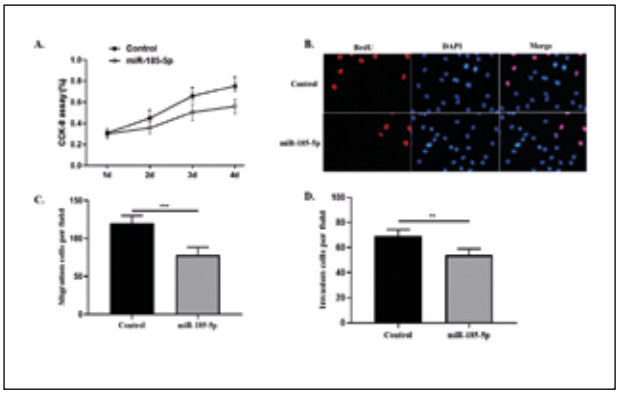

Figure 2: MiR-185-5p overexpression inhibited migration and invasion of CRC cells.

*A. The CCK-8 test was used to detect the proliferation of the control and miR-185-5p-overexpressing SW480 cells at different time points. B. Proliferation of control and miR-185-5p-overexpressing SW480 cells was detected by BrdU immunofluorescence staining. C and D. Migration and invasion levels of control and miR-185-5p-overexpressing SW480 cells were detected by Transwell assay. *, P<0.05; **, P<0.01; ***, P<0.001.*

MiR-185-5p directly targeted TNNT1 3' UTR in CRC cells

In order to further explore the mechanism of TNNT1 imbalance in CRC, the Targetscan database was used to predict both that miR-185-5p may directly target TNNT1 to regulate its expression and the possible binding sequences of the two (Figure 3A). As shown in Fig. 1D, compared with normal cells, the expression level of miR-185-5p in CRC cell lines was significantly downregulated and was negatively correlated with the expression of TNNT1 in CRC. To verify the target relationship between TNNT1 and miR-185-5p, a double luciferase reporter gene test was used, and the results showed that miR-185-5p mimics reduced the luciferase activity of Wt-TNNT1-containing luciferase reporter genes but did not significantly affect the luciferase activity of the Mut-TNNT1 vector (Figure 3A).

This result further confirms the target relationship between the 3' UTR of TNNT1 and miR-185-5p in CRC. At the same time, a miR-185-5p overexpression model was established using the SW480 cell line and the transfection efficiency was verified using RT-qPCR (Figure 3B). Western blotting was used to detect TNNT1 expression after miR-185-5p overexpression, and the results showed that miR-185-5p overexpression significantly reduced TNNT1 expression (Figure 3C). Based on these results, we concluded that the upregulation of TNNT1 in CRC tissues may be due to the imbalance of miR-185-5p.

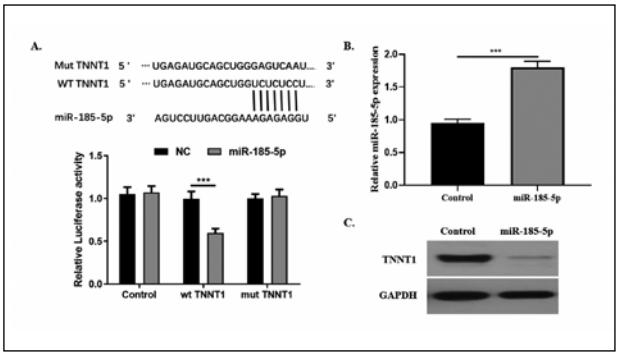

Figure 3: MiR-185-5p directly targets TNNT1 3' UTR in CRC cells.

*A. The binding sequence of miR-185-5p to 3' UTR of TNNT1 was predicted by Targetscan and a double luciferase reporter assay was performed. B. MiR-185-5p expression in control and miR-185-5p-overexpressing SW480 cells was detected by RT-qPCR experiments. C. TNNT1 expression in control and miR-185-5p-overexpressing SW480 cells was detected by Western blotting. ***, p <0.001.*

Forced overexpression of TNNT1 reversed the miR-185-5p-induced inhibition of proliferation and invasion in CRC cells

To further explore the role of the miR-185-5p/TNNT1 axis in CRC, the TNNT1 plasmid was used to forcibly upregulate TNNT1 expression based on miR-185-5p overexpression. First, the expression of miR-185-5p and TNNT1 in each group of transfected cells was detected by RT-qPCR, and the transfection efficiency of each group of cells was tested. Results showed that miR-185-5p overexpression reduced the TNNT1 expression on mRNA levels, and TNNT1 forced overexpression restored the original expression level, while TNNT1 forced overexpression had no effect on the expression level of miR-185-5p (Figures 4A and 4B).

Then BrdU immunofluorescence staining was used to detect the proliferation of SW480 cells transfected in each group. The results showed that forced overexpression of TNNT1 could significantly reverse the inhibitory effect of miR-185-5p on SW480 cell proliferation (Figure C).

The migration and invasion levels of SW480 cells in each group were further tested, and the results showed that compared with the control group, the forced overexpression of TNNT1 also significantly reversed the inhibitory effect of miR-185-5p on the migration and invasion of SW480 cells (Fig-

ures 4D and 4E). These data further confirmed the important role of the miR-185-5p/TNNT1 axis in CRC cells.

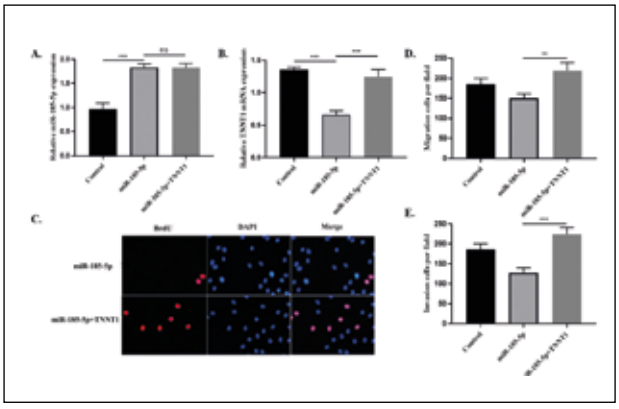

Figure 4: Forced overexpression of TNNT1 reversed miR-185-5p-induced inhibition of CRC cell proliferation and invasion.

A and B. The expression levels of miR-185-5p and TNNT1 in SW480 cells of control group, miR-185-5p overexpression group and TNNT1 forced overexpression group were detected by RT-qPCR. C. The proliferation of SW480 cells in the control group, miR-185-5p overexpression group and TNNT1 forced overexpression group was detected by BrdU immunofluorescence staining. D and E. The Transwell assay was used to detect the migration and invasion levels of SW480 cells in the control group, miR-185-5p overexpression group and TNNT1 forced overexpression group. ***, P<0.01;* ****, P<0.001.*

Discussion

CRC involves a very complex multi-stage pathological process under the control of multiple genes[16], so accurately predicting the prognosis of patients with CRC and choosing an effective treatment plan is of great importance to improving patient survival[17]. The prognosis of CRC may guide individualized treatment to improve the survival rate of patients with CRC[18]. This research showed that TNNT1 expression is upregulated in CRC tissues, suggesting that TNNT1 may be a biomarker of CRC.

Previous studies have reported that mutations in the TNNT1 gene result in the complete loss of TNT in skeletal muscle and eventually lead to severe adrenal myopathy[19]. Surprisingly, research on the role of TNNT1 in regulating tumours has increased in recent years. For example, studies found that the expression of TNNT1 was significantly increased in breast cancer tissues, was closely related to clinical stage and T and N classification, and promoted the proliferation of cancer cells by changing the transformation of G1/S[20]. This study demonstrated that TNNT1 overexpression significantly promotes the proliferation of CRC cells, and the migration and invasion data suggested that TNNT1 has a cancer-promoting role in the development of CRC, similar to that found in breast cancer. Of course, further in vivo experiments are needed in future studies to verify the role of TNNT1 in CRC, and the downstream mechanism of TNNT1 in promoting cancer progression is also worthy of further study.

In recent years, a large number of studies have shown that the 5' end of miRNA can interact with the 3' untranslated region (3' UTR) of messenger RNA (mRNA), which can cause degradation or can inhibit translation through specific pairing, thereby regulating related proteins and downstream signalling pathways, which play a key role in tumour progression[21, 22]. For example, miR-196b-5p regulates the migration and metastasis of CRC cells by interacting with HOXB7 and GALNT5. MiR-103 targets ZO-1 to regulate tumorigenesis of CRC[23], while miR-873-5p targets ZEB1 to inhibit cell migration, invasion and epithelial-mesenchymal transition in CRC[24]. Interestingly, bioinformatics analysis found a targeting relationship between miR-185-5p and TNNT1. Furthermore, we found that TNNT1 promoted the proliferation, migration and invasion of CRC cells, while miR-185-5p inhibited the proliferation, migration and invasion of CRC cells. Luciferase activity assay confirmed the target relationship between TNNT1 and miR-185-5p and found that miR-185-5p may serve as an upstream target molecule for TNNT1 and inhibit the progression of CRC by regulating TNNT1.

In summary, this study found that TNNT1 can promote the proliferation, migration and invasion of CRC cells, suggesting that TNNT1 can be a potential marker for diagnosis and treatment of CRC. In addition, it was found that miR-185-5p has a tumour suppressive effect in CRC cells and negatively regulates TNNT1 to inhibit the progress of CRC. This study explored new mechanisms for the occurrence and development of CRC and provided a new theoretical basis for the diagnosis and treatment of CRC.

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