miR-200c /FUT4 axis prevents the migration, invasion and proliferation of colon cancer cells by downregulating Wnt/β-catenin pathway

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Research article

Keywords: miR-200c, FUT4, colon cancer, Wnt/β-catenin pathway

DOI: https://doi.org/10.21203/rs.3.rs-26901/v1

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Abstract

Background

To investigate the effects of miR-200c targeting fucosyltransferase 4 (FUT4) on the proliferation, migration and invasion of colon cancer cells and further to explore its mechanism.

Methods

The expression of miR-200c and FUT4 mRNA in Lovo and SW480 cells were detected by quantitative real-time polymerase chain reaction (qRT-PCR), and their correlation was analyzed by Pearson. LipofectamineTM 2000 transfection reagent was used to transfect miR-200c mimic, FUT4 siRNA, FUT4 mimic and FUT4 mimic negative control into Lovo and SW480 cells, and RT-PCR was used to analyze the effect of transfection. Cell counting kitcck-8 (CCK-8), cloning and transwell assays were used to detect the migration, invasion and proliferation of Lovo and SW480 cells, respectively. Immunofluorescence was used to analyze the expression of Ki-67 protein. Moreover, the expression of Wnt/β-catenin signaling pathway-related proteins were detected by western blot. Double luciferase experiment was performed to verify the targeting relationship between miR-200c and FUT4.

Results

Pearson results showed that miR-200c and FUT4 were negatively correlated in Lovo and SW480 cells (correlation coefficients were −0.9046 and −0.9236, respectively). MiR-200c overexpression inhibits the proliferation, migration and invasion of Lovo cells by down-regulating FUT4. The expression of Ki67 positive cells and Wnt/β-catenin signaling pathway-related proteins were reduced in miR-200c overexpression and FUT4 silencing groups. The scientific search and dual luciferase reporting system identified FUT4 was the target of miR-200c.

Conclusion

In conclusion, miR-200c overexpression inhibits FUT4 expression and down-regulates the Wnt/β-catenin signaling pathway, thereby inhibiting the migration, invasion and proliferation of colon cancer cells.

Background

Colon cancer, a tumor of the large intestine (colon), is a clinically highly malignant tumor of the digestive tract. Colon cancer ranks third in global gastrointestinal tumors incidence and fourth in mortality. Colon cancer can cause blood in the stool, stomach pain, and change in stool. If it is detected early, most patients with colon cancer can get better. However, there are more than one million new cases of colon
cancer and approximately 700,000 people die of colon cancer each year. In the present, the treatments of colon cancer are not satisfactory.

MicroRNAs (miRNAs) are small and endogenous noncoding RNAs that post-transcriptionally regulate gene expression, which have important functions in plants and animals. Emerging evidence suggests that miRNAs play important roles in promoting or inhibiting tumor cell proliferation, invasion and apoptosis. Moreover, an increasing number of studies have shown that miRNAs are involved in the metastasis of colon cancer. Thus, miRNAs are currently becoming potential targets for colon cancer treatment. The miR-200 family comprises 5 members which located in miR-200a/b/429 and miR-200c/141. Accumulating evidence suggests that miR-200 family is an epigenetic regulator of epithelial-to-mesenchymal transition and involved in cancer progression. Among them, there are many studies on miR-200c and colon cancer, and it has been shown that low expression of miR-200c is associated with poor prognosis of patients. However, the regulation of colon cancer metastasis by miR-200c is a complex biological network. Therefore, the regulatory mechanism of miR-200c on colon cancer metastasis deserves further study.

Wnt/β-catenin signal controls multiple biological phenomena in early life and adult life by regulating cell proliferation and genetic stability. In parallel, aberrant Wnt/β-catenin signaling is associated with a wide a variety of human diseases, such as cancer, osteoporosis, etc. Ghahhari et al. has shown that the miR-200 family not only plays an important role in the regulation of cancer stem cells proliferation and metastasis, but also inhibits migration and invasion of tumor cell by inhibiting Wnt/β-catenin signaling. Therefore, in this work, we investigate the effects of miR-200c on the proliferation, migration and invasion of colon cancer cells. Furthermore, we studied whether its mechanism of action is related to the Wnt/β-catenin signaling pathway. The present study aimed to elucidate the interrelation between miR-200c and Wnt/β-catenin pathway, searching for promising molecular targets to inhibit metastasis for colon cancer therapy.

Methods

Cell culture

Human normal intestinal epithelial cells NCM460 (BNCC353657), human colon cancer cells Lovo (BNCC338601) and SW480 (BNCC288146) were selected and cultured in RPMI-1640 (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA). The cells were all obtained from Shanghai Institute of Cell Research, CAS. The cells were maintained in a humidified cell incubator (Thermo Fisher Scientific, Waltham, USA) atmosphere of 5% CO₂ at 37°C.

Cell groups and transfection

Lovo and SW480 cells were divided into (1) blank control group (BC): no treatment; (2) miR-200c overexpression group: cells were transfected with 100 nM miR-200c mimic lentiviral vector; (3)
fucosyltransferase 4 (FUT4) silencing group (si-FUT4): cells were transfected with 50 nM FUT4 siRNA lentiviral vector; (4) miR-200c + FUT4 overexpression negative control group (miR-200c + NC1): cells were transfected with 100 nM miR-200c overexpression lentiviral vector and 50 nM FUT4 overexpression negative control lentiviral vector; (5) miR-200c + FUT4 overexpression group (miR-200c + FUT4): cells were transfected with 100 nM miR-200c overexpression lentiviral vector and 50 nM FUT4 overexpression lentiviral vectors.

Lovo and SW480 cells in logarithmic growth phase were selected for subsequent experiments. The cells were passaged 1 day before transfection and cultured in a 6-well plate. When the confluence reached 70%, transfection was performed according to the lentiviral transfection instructions. Lentiviral particles were constructed by Shanghai Jikai Biotechnology Co., Ltd (Shanghai, China). miR-200c mimic, mimic-NC, FUT4 siRNA, FUT4 mimic, and FUT4 overexpression negative control lentiviral vectors were purchased from Shanghai GenePharm Pharmaceutical Technology Co., Ltd (Shanghai, China). The expression of miR-200c and FUT4 mRNA in transfected cells was detected by quantitative real-time polymerase chain reaction (qRT-PCR) at 72 h after infection.

**Cell counting kit (CCK)-8 assay**

Logarithmic growth phase cells were inoculated into 96-well plates at a density of $2 \times 10^4$ cells/ml, 100 µl per well. After cultured for 24 h, 48 h, 72 h, and 96 h at 37ºC, 5% CO$_2$ in incubator, 10 µl of CCK-8 solution (Tongren Institute of Chemistry, Japan) was added into each well and incubation for another 4 h. The optical density (OD) of each well at 450 nm was measured by a microplate reader.

**Colony formation assay**

Logarithmic growth phase cells were digested with 0.25% trypsin and adjusted to 250 cells/ml. 2 ml/well cells were cultured in a 6-well plate at 37 ºC, 5% CO$_2$ for 2–3 weeks and the fresh medium was changed every 3 days. Methanol was used to fixed the cells and 1 ml of Ji Giemsa working uid was used to stain the cells for 30 min. After washed twice with ultrapure water, the filter paper was used to suck up the water around the dish and the record was imaged by a camera.

**Transwell assay**

Cell invasion experiment: After digesting, centrifuging and resuspending, the cells were adjusted to $4 \times 10^5$ cells/ml. 50 µl of 1640 medium containing Matrigel (1:1) were added to the transwell upper chamber, and incubated at 37 ºC for 1 h. Then, 100 µl cell suspension was added into the upper compartment of the chamber while 600 µl of complete medium containing 10% FBS was added to the lower chamber. After incubation at 37 ºC and 5% CO$_2$ for 24 h, the membranes were fixed with methanol for 30 min and stained with crystal violet for 15 min. The results were observed under a light microscope (Olympus Corporation) and performed by ImageJ software.

For cell migration experiment, matrigel is not required, and other experimental steps were the same as invasion experiment.
Immunofluorescence

The cells crawling in coverslips were treated differently as required and fixed with 4% paraformaldehyde. After rupturing the membrane with 0.2% Triton X-100, cells were sealed with 5% bovine serum albumin (BSA) and incubated in incubator for 30 min. Then, the cells were incubated with primary antibodies Ki67 (1:200, orb88614, Biorbyt, Cambridge, UK) at 4 °C overnight. After rinsing with phosphate buffer saline (PBS), the cells were incubated with FITC-labeled secondary antibody at 37 °C for 30 min in the dark. Subsequently, the cells were rinsed with PBS, stained with 4’,6-Diamidino-2-Phenylindole (DAPI) and mounted with the glycerol. The fluorescence was observed under an inverted laser confocal microscope (FV1200; New Discovery Technology (China) Co., Ltd, Shanghai, China).

qRT-PCR

Total RNA extraction kit (A27828, MagMAX ™ MiRVana ™ Total RNA Isolation Kit, Thermo Fisher Scientific, Waltham, USA) was used to extract total RNA from the cells. cDNA was synthesized by the reverse transcription kit (Applied Biosystems, Waltham, MA, USA) and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA USA) was used for RT-PCR. The miR-200c primer was synthesized by Shanghai Shengong Biotechnology Co., Ltd. (Shanghai, China), and the reaction was performed under the following conditions (40 cycles): 95 °C for 10 min, 95 °C for 15 s, and 60 °C for 1 min. The miR-200c and FUT4 mRNA were internally referenced with U6 and GAPDH, respectively, and the data was processed by the $2^{\Delta \Delta Ct}$ method. The sequence of the primers are as follows: MiR-200c, Forward: 5’-CCTATGTAAACAGCCTCGACTG-3’ and Reverse: 5’-CTGGCGTATCGTGAGTCG-3’. U6, Forward: 5’-GACCTCTATGCCAACACAGT-3’ and Reverse: 5’-AGTACTTGCGCTCAGGAGGA-3’. FUT4, Forward: 5’-AAGGTCCAGGCCACTGAAG-3’ and Reverse: 5’-CAGTTCAAGGTGACAGAGGCTCA-3’. GAPDH, Forward: 5’-ATGGGGAAGGTGAAGGTCG-3’ and Reverse: 5’-GGGGTCATTGATGGCAACAATA-3’.

Western blot

After lysed and centrifuged, the protein concentration of the cells was measured by BCA kit (Solarbio, Beijing, China). Then, the protein samples were transferred to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis (Mini-Protean-3, Bio-Rad, Hercules, CA, USA) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Massachusetts, USA). After blocking with 5% skim milk, the membranes were incubated with primary rabbit anti-human antibodies against β-catenin (1:2000, ab16051, Abcam), CyclinD1 (1:200, ab16663, Abcam), GSK-3β (1:5000, ab32391, Abcam), p-GSK-3β (1:1000, ab131097, Abcam), GAPDH (1:2500, ab9485, Abcam, UK), and mouse anti-human FUT4 (1:1000, sc-19648, Santa Cruz Biotechnology). After washed three times with TBST (TBS, 1 ml/L Tween-20), the members were incubated with horseradish peroxidase-conjugated goat anti-rabbit (1:2000, ab6721, Abcam) and goat anti-mouse (1:2000, sc-2354, Santa Cruz Biotechnology) immunoglobulin G secondary antibodies. Finally, the enhanced chemiluminescence (ECL) chemiluminescence method was used for detecting signals, and the grayscale scanning and quantification of the protein band were performed by Image J (NIH) software. The expression levels of proteins were normalized to GAPDH.
Double luciferase reporter assay

Wild type and mutant 3'UTRs of FUT4 were amplified in pGL3/luciferase vector (Promega, Madison, WI, USA) and cloned downstream of the luciferase gene. The luciferase activity of the cells was detected with the dual luciferase reporter system (Promega) at 48 h after transfection according to the instructions.

Statistical analysis

SPSS 19.0 statistical analysis software was used for data processing, and the results of data analysis were expressed as mean ± standard deviation (mean ± SD). The t-test was exerted for data analysis between two groups, and one-way analysis of variance (ANOVA) was used for data analysis of multiple-group comparison. The difference was statistically significant at p < 0.05.

Results

The mRNA expression of miR-200c and FUT4 in colon cancer cells

As can be seen from Fig. 1, the expression of miR-200c mRNA in Lovo and SW480 cells was significantly lower than that of NCM460 cells (p < 0.05), and the expression of FUT4 mRNA in Lovo and SW480 cells was significantly higher than that of NCM460 cells (p < 0.05). Pearson analysis showed a negative correlation between the mRNA expression of miR-200c and FUT4 (r = -0.9046 for Lovo cells and r = -0.9236 for SW480 cells).

MiR-200c overexpression inhibits proliferation of Lovo and SW480 cells by down-regulating FUT4

The results of Fig. 2A and 2B showed that the expression of miR-200c mRNA in the miR-200c group, si-FUT4 group, and miR-200c + NC1 group were significantly higher than those in BC group (p < 0.05), while FUT4 mRNA expression showed opposite trend. Compared with miR-200c group and miR-200c + NC1 group, the expression of the miR-200c mRNA in miR-200c + FUT4 group was significantly reduced (p < 0.05), while FUT4 mRNA was evidently increased (p < 0.05). Moreover, compared with BC group, there was a significant decrease in the expression of FUT4 mRNA in miR-200c group. Those results indicated that miR-200c can down-regulate the expression of FUT4.

CCK-8 (Fig. 2C) and colony formation assay (Fig. 2D) showed that the proliferation of Lovo and SW480 cells in miR-200c group, si-FUT4 group, and miR-200c + NC1 group were dramatically decreased than that of the BC group (p < 0.05). Meanwhile, compared with 200c group and miR-200c + NC1 group, the proliferation of Lovo and SW480 cells in miR-200c + FUT4 group was significantly increased (p < 0.05). All these findings suggested that overexpression of miR-200c could inhibit the proliferation of colon cancer cells by down-regulating FUT4.
MiR-200c overexpression inhibits migration and invasion of Lovo and SW480 cells by down-regulating FUT4

As shown in Fig. 3, the invasion and migration of Lovo and SW480 cells in miR-200c group, si-FUT4 group, and miR-200c + NC1 group were evidently decreased than that in BC group (p < 0.05). Simultaneously, compared with miR-200c group and miR-200c + NC1 group, the invasion and migration of Lovo and SW480 cells in miR-200c + FUT4 group was significantly increased (p < 0.05).

MiR-200c overexpression inhibits the expression of Ki67 in Lovo and SW480 cells by down-regulating FUT4

As exhibited in Fig. 4, compared with BC group, miR-200c or si-FUT4 can significantly inhibit the expression of Ki67 positive in Lovo and SW480 cells (p < 0.05). Furthermore, compared with miR-200c group and miR-200c + NC1 group, the expression of Ki67 was significantly increased after co-transfection of miR-200c and FUT4 (p < 0.05). The results indicated that miR-200c overexpression could inhibit the expression of Ki67 in colon cancer cells by down-regulating FUT4.

MiR-200c overexpression inhibits the expression of Wnt/β-catenin-related proteins by down-regulating FUT4

Figure 5 showed that compared with BC group, the expression of Ki-67, FUT4, β-catenin, CyclinD1, and p-GSK-3β in miR-200c group and si-FUT4 group were significantly down-regulated (p < 0.05). For miR-200c + FUT4 group, simultaneously treatment with miR-200c and FUT4 was evidently up-regulated the expression of the above proteins (p < 0.05), which indicated that miR-200c overexpression inhibits the expression of Wnt/β-catenin-related proteins by down-regulating FUT4.

FUT4 is the target of miR-200c

As showed in Fig. 6, a bioinformatics search was used to determine that FUT4 was the target of miR-200c. To further verify whether miR-200c targets FUT4, a dual luciferase reporting system was used. The results showed that miR-200c could reduce the luciferase activity of FUT4 containing WT 3’UTR, but did not decrease the luciferase activity of FUT4 containing Mut 3’UTR.

Discussions

In the past few decades, patients with colon cancer have increased rapidly in the world, especially those over 50 years of age. In order to improve the quality of life of the elderly, it is urgently to develop effective therapeutic approaches for colon cancer. As a member of miRNA family, miR-200 affects metastasis in some type of cancers. In this study, the regulatory mechanism of miR-200c on colon cancer metastasis deserves was firstly evaluated. We found that the mRNA expression of miR-200c in Lovo and
SW480 cells was remarkably lower than that in NCM460 cells, and the miR-200c overexpression treatment could inhibit proliferation of Lovo and SW480 cells. This indicated that miR-200c plays a positive role in the treatment of colon cancer, which is consistent with previous research results.\textsuperscript{11–13}

Tumors are essentially a polygenic disease in which cells escape normal growth control mechanisms and undergo autonomic nerve proliferation.\textsuperscript{18} The tumor cells become invasive and are activated by one or more proto-oncogenes or tumor suppressor genes.\textsuperscript{18} Through predicted by databases and dual luciferase assay, we found that FUT4 is the target of miR-200c. FUT family is a class of glycosyltransferase molecules that are involved in the synthesis of glycoproteins and glycolipid sugar chains on the cell surface, which play important role in a variety of physiological processes.\textsuperscript{19} The FUT family can be divided into four subfamilies according to the different transferase activities. FUT4 is a key enzyme for the synthesis of sialylated Lewis oligosaccharide X (SLeX), and the previous study showed that abnormal expression of FUT4 has important links with tumorigenesis, invasion and metastasis.\textsuperscript{20}

The mechanisms involved in the miR-200c on colon cancer have not yet been completely elucidated. As we all know, cell proliferation is the foundation of organism growth, development, reproduction, and heredity.\textsuperscript{21} Uncontrolled proliferation, migration and invasion provide a survival advantage of cancer cells to resist conventional chemotherapeutic agents.\textsuperscript{22} In the present study, CCK-8 and cloning formation assays confirmed the miR-200c overexpression could inhibit the proliferation of Lovo and SW480 cells by targeting FUT4. Furthermore, transwell and immunofluorescence assays suggested that miR-200c overexpression could inhibit the invasion and migration of Lovo and SW480 cells by targeting FUT4. Wnt signaling pathway is widely present in invertebrates and vertebrates, and is a class of highly conserved signaling pathways during species evolution. A number of studies have shown that Wnt/β-catenin signaling pathway is associated with a wide a variety of human diseases, and miR-200 family can inhibit tumor cell migration and invasion by inhibiting Wnt/β-catenin signaling.\textsuperscript{14–16} In this study, the protein expression of Ki-67, FUT4, β-catenin, CyclinD1, and p-GSK-3β were down-regulated after miR-381 overexpression or silencing FUT4. However, simultaneously treatment with miR-200c and FUT4 was evidently up-regulated the expression of the above proteins.

**Conclusion**

In conclusion, our study shown that miR-200c overexpression can alleviate the colon cancer. The mechanism may be related to the inhibition of FUT4 expression and down-regulation of Wnt/β-catenin signaling pathway, thereby inhibiting the migration, invasion and proliferation of colon cancer cells.

**Abbreviations**

Not applicable

**Declarations**
All manuscripts must contain the following sections under the heading 'Declarations':

**Ethics approval and consent to participate**

Animal experiments were followed the NIH guidelines (NIH Pub. No. 85 – 23, revised 1996) and have been approved by the Animal Protection and Use Committee of Shengjing Hospital, China Medical University.

**Consent for publication**

Not applicable

**Availability of data and materials**

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

**Competing interests**

The authors declare that they have no competing interests

**Funding**

This study was supported by Natural Science Foundation Guidance Program of Liaoning Province( 2019-ZD-0747).

**Authors' contributions**

JC and CY carried out the experimental work and the data collection and interpretation. JG and JC participated in the design and coordination of experimental work, and acquisition of data. ZX and JC participated in the study design, data collection, analysis of data and preparation of the manuscript. JC and HZ carried out the study design, the analysis and interpretation of data and drafted the manuscript. All authors read and approved the final manuscript.

**Acknowledgements**

Not applicable

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Figures
Figure 1

The mRNA expression of miR-200c and FUT4 in colon cancer cells. (A) The mRNA expression of miR-200c and FUT4 in Lovo cells; (B) The mRNA expression of miR-200c and FUT4 in SW480 cells; (C) Pearson analysis of the correlation between miR-200c and FUT4 in Lovo and SW480 cells
Figure 2

MiR-200c overexpression inhibits proliferation of colon cancer cells by down-regulating FUT4. (A) The transfection efficiency of miR-200c and FUT4 in Lovo cells; (B) The transfection efficiency of miR-200c and FUT4 in SW480 cells; (C) CCK-8 assay; (D) Cloning formation assay. *p < 0.05 vs. BC group; #p < 0.05 vs. miR-200c; &p < 0.05 vs. miR-200c + FUT4 group
Figure 3

Transwell assay was used to analyze the effect of miR-200c overexpression on the migration and invasion of colon cancer cells (×400). *p < 0.05 vs. BC group; #p < 0.05 vs. miR-200c; &p < 0.05 vs. miR-200c + FUT4 group

Figure 4

Immunofluorescence was used to analyze the effect of miR-200c overexpression on the expression of Ki67 in colon cancer cells (×400). *p < 0.05 vs. BC group; #p < 0.05 vs. miR-200c; &p < 0.05 vs. miR-200c + FUT4 group
Figure 5

Western blot was used to detect the effect of miR-200c overexpression on the expression of Wnt/β-catenin-related proteins. *p < 0.05 vs. BC group; #p < 0.05 vs. miR-200c; &p < 0.05 vs. miR-200c + FUT4 group
Figure 6

FUT4 is a downstream target gene of miR-200c. (A) Mutations were generated in the 3'UTR sequences of the FUT4 mRNAs at the complementary sites for the seed regions in miR-200c; (B) Dual luciferase experiment was used to analysis the effect of miR-200c overexpression on the activity of FUT4 wild-type and mutant 3'UTR. *p < 0.05 vs. BC group