Original Article

Effect of long-chain non-coding RNA H19 targeting Mir-17 on invasion and migration of colon cancer

Zhi-Ning Liu, Lian-Bang Zhou, Heng Jiang, Sheng-Yun Wan, Yong Wang, Gang Yu

Department of General Surgery, The Second Hospital of Anhui Medical University, Hefei, Anhui Province, China

Received March 10, 2017; Accepted May 9, 2017; Epub June 1, 2017; Published June 15, 2017

Abstract: Objective: To investigate the expression of H19 in colon cancer and the role and mechanism of H19 in invasion and migration of colon cancer cells. Methods: The expression of H19 in colon cancer and adjacent tissues and different colon cancer cells was detected by qPCR. Transwell invasion assay was used to detect the invasion ability of colon cancer cells after silencing H19. The migration ability of colon cancer cells after silencing H19 was detected by scratch test the expression of miR-17 in colorectal cancer and paracancerous tissues was detected by qPCR. Transwell invasion assay was used to detect the expression of miR-17 in silenced H19 cells. The expression of miR-17 was detected by double-luciferase reporter gene. The effect of miR-17 on the proliferation of colon cancer cells after silencing H19 was detected by plate cloning assay. The effect of miR-17 on the proliferation of colon cancer cells was observed by scratch test. The effect of miR-17 on the tumor size and volume of colon cancer was detected after silencing H19. Western blotting was used to detect the expression of Notch pathway protein after silencing H19. Results: The expression of H19 in colorectal cancer cells was significantly higher than that in adjacent tissues, and the expression of H19 in colon cancer cells SW116 was the highest. Silencing H19 could inhibit the invasion and migration of colon cancer cells. H19 could be associated with 3RU of miR-17 specifically. The expression of miR-17 was significantly increased in colon cancer tissues compared with adjacent tissues. Overexpression of miR-17 could promote the proliferation, invasion and migration of colon cancer cells after silencing H19; the tumor volume and weight of H19-siRNA + miR-17-mimic group were significantly increased compared with H19-siRNA group. The expression of miR-17 and Notch pathway protein was up-regulated after silencing H19. Conclusion: H19 plays an important role in the development and progression of colon cancer. It can target the regulation of miR-17 through Notch signaling pathway to regulate the proliferation, invasion and migration of colon cancer cells.

Keywords: H19, colon cancer, miR-17, transwell

Introduction

Colon cancer is one of the most common malignant tumors of the digestive tract; the incidence of colon cancer gradually increased with the change in people’s eating habits, high fat and high protein intake increased, especially in developing countries. The incidence and mortality of colorectal cancer in China accounted for the third and fifth [1, 2] at present. Many researchers have shown that colon cancer is a multi-stage, multi-factor, multi-gene and signal pathway involved in the process [3]. Early colon cancer generally did not show obvious symptoms, patients with obvious symptoms are often in the middle and late period. The prognosis of patients with colon cancer has improved significantly with the rapid development of surgery based comprehensive treatment in recent years. Researches have shown that 30-40% of patients have had local infiltration and peripheral metastases at the time of treatment [5]. The invasion and metastasis of colon cancer are the main causes of death, but the mechanism of tumor invasion and metastasis is not clear, so the mechanism of invasion and metastasis of colon cancer and related molecular target research is the focus of colon cancer research.

Long-non-coding RNAs (lncRNAs) are RNAs of more than 200 nt transcripts; this class of RNA itself does not encode the protein, but regulate the expression level of the gene, and can be used as a modified chromatin complex scaffold [6]. Many researchers have found that long-chain non-coding RNA H19 is expressed abnormally in a variety of tumors, such as gastric can-
cancer, bladder cancer, etc. H19 was found to have cytostatic activity in these malignancies, but researches have suggested that H19 also has potentiating activity [7-9], which can inhibit tumor proliferation, invasion and migration, so the biological function and function of H19 in colon cancer and its mechanism need to be further investigated. MicroRNA is a kind of evolutionarily highly conserved endogenous small fragment of non-coding single-stranded RNA. The expression of target gene is changed by binding to the target gene mRNA 3’UTR, which is an important molecule for post-transcriptional regulation of gene expression. MiRNAs have a very important role in various cell life activities such as self-replication, proliferation, differentiation, apoptosis, etc. [11]. Many researchers have shown that miRNA abnormal expression is closely related to human tumors, miR-17 in colorectal cancer in high expression, but miR-17 in colon cancer and its specific mechanism is not clear.

This research was to investigate the expression of H19 in colon cancer, and to further explore the interaction between H19 and miR-17 and its correlation, and to clarify the role of H19 in the migration and invasion of colon cancer.

Materials and methods

Samples collection

125 cases of colon cancer patients with tumor tissue and paracancerous tissue admitted to our hospital from March 2015 to May 2016 were collected. All patients had no chemotherapy or radiotherapy before surgery; pathological sections were confirmed by two pathologists. There were 27 cases of grade A, 51 cases of grade B, 31 cases of grade C, 16 cases of grade D, 25 cases of poorly differentiated, 72 cases of poorly differentiated and 28 cases of well differentiated according to the Dukes stage of colon cancer. 31 cases had lymph node metastasis, 94 cases had no lymph node metastasis. The tumor tissue was quickly put into RNA preservation solution.

Cell lines

Human colon cancer cells SW116, HT-29, HCT116, SW480 were purchased from the Wuhan Cell Collection, and cultured in DMEM medium containing 10% fetal bovine serum in 37°C, 5% CO₂ incubator to cultivate and passage. Fetal bovine serum, RPMI 1640 medium were purchased from Hyclone Corporation (Hyclone, Logan, UT). Transwell chamber was purchased from Millipore (Millipore, Billerica, MA); Matrigel was purchased from Bio-Rad (Bio-Rad, Madrid, Spain). Lipofectamine 2000, miR-17-inhibitor was purchased from (Gnenpharma Co., Shanghai, China). Trierol was purchased from Ambion (Ambion Inc., Austin, TX, USA), reverse transcription kit (FSQ-101) was purchased from Japan TOYOBO Corporation (TOYOBO, FSQ-101, Japan), PCR kit was purchased from Sigma (KapaBiosystems Inc., Boston, US). The luciferase activity assay kit was purchased from Promega (Promega Biotech Co., Beijing, China). The luciferase reporter vector was synthesized by Promega Corporation (Promega Biotech Co., Beijing, China).

Quantitative real-time polymerase chain reaction

The expression of H19 was detected by immunohistochemistry. The cells were inoculated into the culture flask at a dose of 1 × 10⁵/L. After 36 hours of culture, the total RNA was extracted according to Trizol. The concentration and purity of the nucleic acid were detected by UV spectrophotometer. H19 upstream primer: 5’-GCTTTGAGCGTTGGATCT-3’, downstream primer: 5’-CTCGATCTTCTATCTAGAGTT-3’. MiR-17 upstream primer: 5’-GCCGCAAGTGCTTACAGT-3’, downstream primer: 5’-TGGAGGTCAGGTAT-3’. The total RNA was diluted to the same concentration with DEPC, the reagents were added as described, and the cDNA was stored at -20°C after completion of the reaction. The reaction system was prepared according to the operating instructions of the United States Sigma System, and the volume of each reaction system was 20 μL, and three wells were set, each group of samples was required to carry out the target RNA and internal reference gene GAPDH fluorescence quantitative PCR amplification. According to ΔΔCT method, the reaction conditions were as follows: 37°C 15 min, 98°C 5 min. Followed by
Effect of rnah19 targeting Mir on the invasion and migration of colon cancer

PCR reaction according to the PCR kit instructions. Obtain the data to calculate the mRNA expression by RQ = 2^-ΔΔCT.

Cell transfection

The cells were adjusted to logarithmic growth state and were seeded on 24-well plates at 36 h before transfection; the cell density reached 50% to 80% at day 2. The virus was set on ice and then diluted with the best MOI solution. 5 ng/mL of polybrene was then mixed gently into the cells. The miR-17-inhibitor and negative control cells were transfected according to the Lipofectamine 2000 Transfection Kit. The complete medium was replaced after incubation in the incubator for 12 h.

Transwell invasion assays

The Transwell chamber used in this experiment was purchased from BD, with a cell aperture of 8 μm. Matrigel was spoiled overnight at 4°C and the Matrigel gum was diluted in a 1:8 ratio in a serum-free pre-cooled 1640 medium. Matrigel was diluted with 100 μL and added to the Transwell chamber. The cells were placed in 24-well plates and incubated at 37°C for 4 h so that it was gel-like, take the logarithmic group of cells, with serum-free medium adjusted to 1 × 10^6/ml cell suspension, take 0.2 ml into the upper chamber, the next room by adding 0.6 ml containing 10% fetal cattle. The cells were washed with 0.1% crystal violet for 2 minutes, and the cells were stained with 0.1% crystal violet for 2 minutes. The cells were washed with 0.1% crystal violet for 2 minutes, and the cells were washed with 0.1% crystal violet for 2 minutes. Buffer washed twice, observed under an inverted microscope and photographed, each experiment repeated 3 times.

Wound-healing assays

The logarithmic phase cells were seeded in 6 well plates, cell density of 5 × 10^4/ml, when cell wall into monolayer cells after disinfection with a 10 uL gun head uniform draw 4 lines, with PBS away draw the cells into culture medium containing 1.5% fetal bovine serum culture, set three complex the hole, placed in 5% CO₂ incubation. Photographed under a microscope according to the experimental design of sampling time point. The initial distance measuring scratch was (0 time); Scratches were measured and photographed after 24, 48, 72 h, distance migration rates were calculated. The test was repeated 3 times.

Luciferase activity assays

The luciferase reporter vector was co-transfect ed with H19-siRNA to colon cancer cells. The transfected pRL-TK was used as standard internal control. The cells were harvested after transfection for 36 h. The luciferase activity of colon cancer cells was detected by Promega's luciferase activity assay kit. Calculate relative luciferase activity = firefly luciferase activity value/bloody luciferase activity value.

Western blotting assays

Cells were extracted from each group using the whole protein extraction kit and quantified. SDS-PAGE electrophoresis was performed on 50 μg of total protein per well. Then, the protein was transferred onto PVDF membrane and blocked with TBST containing 5% skimmed milk powder for 2 hours. Added the antibody to the overnight anti- °C incubation 1 h after washing the membrane, washed film, ECL luminescence, Bio-Rad gel imaging system to collect images. Quantity one software was used for gray value analysis, and each experiment was repeated 3 times.

Plate cloning assays

Monolayer cells in logarithmic growth phase were digested with 0.25% trypsin and beaten into individual cells, and the cells were sus pended in RPMI 1640 medium of 10% fetal bovine serum. The cell suspension was diluted in a gradient fold and seeded in a Petri dish at the appropriate cell density (depending on the ability to proliferate). Generally, the gradient density of 50, 100, 200 cells was inoculated with 10 mL of 37°C pre-warmed culture medium and gently rotated to disperse the cells evenly. It was placed in 37°C, 5% CO₂ and saturated humidity environment, standing culture for 2 to 3 weeks. The culture was terminated when clones visible to the naked eye were present in the culture dish. Supernatant was discarded; PBS was washed, fixed with pure methanol for 15 min, crystal violet for 15 min, washed with water, and air dried. The clonal count was calculated by cloning the number of clones/inoculated cells × 100%.
Effect of rnah19 targeting Mir on the invasion and migration of colon cancer

Colon cancer xenografts

The colon cancer SW116 cells were transfected with H19-siRNA and H19-siRNA + miR-17-inhibitor to 80-90%, respectively. The cells were made into a cell suspension with a cell concentration of $2 \times 10^6$/ml. Nude mice are selected from 4 to 6 weeks old. Take 0.1 ml cell suspension injection in each nude mice left forelimb axillary subcutaneous, a total of 10, each group of 5. The survival rate, body weight and survival status of the mice were monitored within 4 weeks after injection. The nude mice were sacrificed and the tumor was taken out after 28 days. The size and weight of the tumor were measured.

Statistical analysis

SPSS 21.0 software was used for statistical analysis. The data were expressed as mean ± standard deviation. The measurement data were analyzed by one-way ANOVA. The variance was treated with Karuskal-Wallis method. The difference was considered statistically significant at $P<0.05$.

Results

Expression of H19 mRNA and miR-17 mRNA in colon cancer tissue and adjacent tissues and expression of H19 mRNA in colon cancer cells

The results of QPCR showed that (Figure 1A) the expression of H19 mRNA in colon cancer tissues was significantly higher than that in adjacent tissues ($P<0.05$), and the difference was statistically significant ($P<0.05$). The expression level of H19 in SW116 cells was the highest in different colon cancer cells (Figure 1B). The expression of miR-17 mRNA in colon cancer tissues was significantly higher than that in adjacent tissues (Figure 1C), the difference

Table 1. Relationship between H19 expression and clinicopathological features of colon cancer

<table>
<thead>
<tr>
<th>Clinicopathologic data</th>
<th>Quantity</th>
<th>H19 low expression</th>
<th>H19 high expression</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>75</td>
<td>36</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>50</td>
<td>25</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td><strong>Year (y)</strong></td>
<td>0.686</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤60</td>
<td>55</td>
<td>27</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>&gt;60</td>
<td>70</td>
<td>37</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td><strong>Polarization</strong></td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>28</td>
<td>22</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Middle</td>
<td>72</td>
<td>38</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>25</td>
<td>3</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td><strong>Pathological stage</strong></td>
<td>0.005</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>27</td>
<td>21</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>51</td>
<td>26</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>31</td>
<td>8</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>16</td>
<td>2</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td><strong>Lymphatic metastasis</strong></td>
<td>0.002</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>94</td>
<td>45</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>31</td>
<td>3</td>
<td>28</td>
<td></td>
</tr>
</tbody>
</table>
Effect of rnah19 targeting Mir on the invasion and migration of colon cancer

was statistically significant. These results suggested that H19 and miR-17 are highly expressed in colon cancer. H19 and miR-17 played an important role in colorectal cancer. We selected colon cancer SW116 cells as further experimental cell lines.

Relationship between H19 expression and clinicopathological data of colon cancer

Statistical analysis showed that the expression level of H19 increased with the increase of pathological stage of colon cancer (Table 1). The expression level of H19 increased gradually with the decrease of differentiation degree. The expression of H19 was significantly higher in colorectal carcinoma with the increase of lymph node metastasis; H19 expression level has nothing to do with age and gender. The results showed that H19 was associated with pathological stage of colon cancer and lymph node metastasis, but not with sex, age and so on.

Effect of silencing H19 on invasion and migration of human colon cancer cell line SW116

Transwell results showed that: (Figure 2A) H19-siRNA group colon cancer cells SW116 Matrigel matrix by the number of cells was significantly increased compared with the NC group, the difference was statistically significant (P<0.01), which indicated that H19-siRNA can inhibit the invasion of human colon cancer SW116 cells.
Effect of rnah19 targeting Mir on the invasion and migration of colon cancer

The results of scratches showed that the mobility of H19-siRNA group was significantly lower than that of NC group at 24 h, 48 h and 72 h (P<0.01), indicating that H19-siRNA can inhibit the migration of human colon cancer SW116 cells.

Relationship between H19 and miR-17 was detected by luciferase reporter gene

The use of bioinformatics predictors suggests that H19 may interact directly with miR-17 in order to clarify the situation of miRNAs associated with H19. To demonstrate whether H19 binds to miR-17 3'UTR, we treated H19-siRNA with miR-17 co-transfected into colon cancer SW116 cells. Luciferase reporter gene results showed (Figure 3) that H19 significantly inhibited luciferase activity in miR-17. The results show that H19 could specifically bind to the 3'UTR of miR-17.

Effects of miR-17 on the invasion and migration of human colon cancer SW116 cells after silencing H19

To clarify the effect of miR-17 on the invasion and migration of colon cancer cells, we used miR-17-mimic to overexpress miR-17 expression, and to investigate the role of miR-17 in the invasion and migration of colon cancer cells. The Transwell results (Figure 4A) showed that the number of cells in the H19-siRNA + miR-17-inhibitor group was significantly increased by Matrigel matrix gel (P<0.01), indicating that overexpression of miR-17 can promote the invasion of human colon cancer SW116 cells after silencing H19.

Effect of miR-17 on the proliferation of colon cancer cells after silencing H19 detected by plate cloning

The results of plate cloning showed (Figure 5A) that the number and size of H19-siRNA + miR-17-mimic cell clones were significantly increased compared with H19-siRNA group, so the effect of H19 on colony cancer cell clone formation was dependent on the expression of miR-17.

Expression of Notch1, NICD1 and Hes1 promoted by silencing H19 and overexpression of miR-17

Researches have shown that Notch signaling pathways played a very important role in tumor invasion and migration. When Notch was activated, it was enzymatically digested to form water-soluble NICD, which bind to the DNA binding protein CSL on the nucleus to form a transcription factor, thereby stimulating the transcription of the target gene Hes1 [12].

Western blotting showed that the expression of Notch1, NICD1 and Hes1 protein in H19-siRNA + miR-17-mimic group was significantly higher than that in H19-siRNA group, and the difference was statistically significant (P<0.05), indicating that silencing H19, overexpression of miR-17 can promote the expression of Notch1, NICD1 and Hes1 and indicating that H19 and miR-17 can function through the Notch signaling pathway.

Impact of miR-17 on tumor growth detected in nude mice subcutaneous tumor after silencing H19

Subcutaneous tumor 28 days after the death of the neck of the nude mice, the autopsy showed that the left armpit tumor growth, the tumor was gray white, solid, round or oval, the surface of nodular protrusions, profiles of fish samples, the rate of 100%.
Effect of rnah19 targeting Mir on the invasion and migration of colon cancer

Nude mice tumor growth (**Figure 6A**): the tumor size of H19-siRNA + miR-17-mimic group was significantly higher than that of H19-siRNA group. The difference was statistically significant (P<0.05). Comparison of tumor weight and volume (**Figure 6B, 6C**): the tumor volume and weight of H19-siRNA + miR-17-mimic group were significantly higher than those of H19-siRNA group. The difference was statistically significant (P<0.05).

**Discussion**

LncRNA has been considered to be no function, the researchers found more and more non-coding RNA with the development of experimental technology, especially the development of sequencing technology. The length of more than 200 bp plays a very important role in the transcriptional regulation process. LncRNA as an important non-coding RNA, many molecular biological functions can be revealed. Researches have shown that lncRNAs can be closely related to epigenetic modifications, transcriptional regulation, protein translation, and protein degradation [13, 14].

LncRNA H19 is transcribed by the H19 gene and is a lncRNA that is maternal in human tissue [15]. H10 has been found to be closely related to the process of emergence, development, invasion and migration of tumors. How-
ever, the specific mechanism of action in tumor is not clear. In different tumors, it may play a role through tumor suppressor or proto-oncogene function. In this investigation, the expression of H19 in colorectal cancer tissues and adjacent tissues was significantly higher than that in colorectal cancer tissues. The expression of H19 in colorectal carcinoma was significantly higher than that in clinicopathological data. It was found that H19 was positively correlated with pathological stage of colon cancer and lymph node metastasis, suggesting that H19 plays an important role in the development and progression of colon cancer.

Researches have shown that the expression of H19 increased, which can lead to a variety of tumor cells to enhance the invasion and migration capacity. Leighton et al. [16] studies
have shown that H19 expression level and lung cancer staging, and H19 and lung cancer invasion depth and metastasis range was related. In this investigation, we found that the invasion and migration ability of colon cancer cells were weakened by silencing H19. The results of previous experiments indicated that H19 played an important role in the development and progression of colon cancer.

The research of LncRNA including bioinformatics needs to be combined with experimental validation and biochip screening combined with experimental validation at present. In this investigation, bioinformatics analysis confirmed that H19 and miR-17 have a direct interaction, further through the double luciferase reporter gene confirmed that H19 can be combined with miR-17 3'UTR. The expression level of miR-17 in colorectal cancer tissues and adjacent tissues was detected and we found that miR-17 expression in colon cancer was significantly higher compared with the adjacent tissues. The miR-17~92 cluster is the first with the role of oncogene miRNA [17], consistent with the results of this investigation.

To further investigate the role of H19 and miR-17 in the biological function of colon cancer and the related mechanism, we used silencing H19, overexpressing miR-17 expression to further investigate the miR-17 in colon cancer cell invasion and migration process effect. The results showed that the expression of miR-17 overexpressing H19 could promote the proliferation, invasion and migration of colon cancer cells. Similar results were observed in vivo experiments using nude mice subcutaneously.

Notch signaling pathway is a highly conserved signaling pathway in the process of biological evolution. Notch signaling pathway is closely related to biological behavior such as tumor proliferation, apoptosis, invasion and migration in the tumor. Yun et al. [18] found that MCF-7/ADR had stronger ability to invade and migrate than MCF-7 compared to MCF-7/ADR while Notch-1 expression level is higher, indicating that the invasion and migration of breast cancer cells are associated with Notch-1. Jie et al. [19] found that Notch-1 expression was significantly increased with the progress of liver cancer staging by analyzing the different stages of liver cancer tissue and the high expression of Notch-1 is closely related to lymph node metastasis and distant metastasis. This investigation found that overexpression of miR-17 after silencing H19 revealed that overexpression of miR-17 could promote the expression of Notch1, NICD1 and Hes1, indicating that H19 and miR-17 may function through the Notch signaling pathway.

The expression of H19 and miR-17 in colon and adjacent tissues was detected by qPCR in this investigation. The relationship between H19 and miR-17 was further observed, and the effects of H19 and miR-17 on the proliferation, invasion and migration of colon cancer cells were further investigated. Researches have shown that H19 and miR-17 are up-regulated in colon cancer and H19 has a direct interaction with miR-17. H19 can target the invasion and migration of colon cancer by miR-17. H19 can regulate the expression of Notch1, NICD1 and Hes1, indicating indirectly that Notch signaling pathway plays a role in H19 regulation of miR-17 affect the biological function of colon cancer cells. It suggests that H19 and miR-17 may be involved in the proliferation, invasion and migration of colon cancer cells, which may be a marker for predicting the progress of colon cancer, prognosis and monitoring of therapeutic effects.

Acknowledgements

Anhui Provincial Health Department Clinical Medicine Application Technology Project (20-08B070).

Disclosure of conflict of interest

None.

Address correspondence to: Lian-Bang Zhou, Department of General Surgery, The Second Hospital of Anhui Medical University, 678 Fu Rong Road, Hefei, Anhui Province, China. Tel: +86-1515606-0699; E-mail: doctorll22@163.com

References

Effect of rnah19 targeting Mir on the invasion and migration of colon cancer


