Review Article

Overexpression of MiR-138-5p targeting SIRT1 mediates PI3K/AKT signaling pathway to promote the proliferation and invasion of colon cancer cells

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Abstract: Objective: The aim of this study was to explore the expression and related mechanisms of miR-138-5p and NAD-dependent protein deacetylase sirtuin-1 (SIRT1) in colon cancer (CC). Methods: One hundred and seven CC patients (CC group) and eighty healthy subjects (normal group) were collected. MiR-NC, miR-138-5p-mimics, miR-138-5p-inhibitor, si-SIRT1, and Sh-SIRT1 were transfected into SW620, HCT116 of the purchased CC cells. qRT-PCR was employed to detect the miR-138-5p and SIRT1 levels in the samples, and phosphatidylinostol 3-Kinase (PI3K), protein kinase B (AKT), p-Akt and SIRT1 in cells were measured by WB. MTT and Transwell were adopted to detect cell proliferation and invasion. Results: MiR-138-5p was lowly expressed in the patient’s serum, while SIRT1 was highly expressed, both of which had an area under the curve (AUC) of >0.9. MiR-138-5p and SIRT1 were associated with Dukes staging, tumor diameter, lymph node metastasis and differentiation degree of CC patients. Overexpressed miR-138-5p and inhibited SIRT1 expression could inhibit cell proliferation and invasion. WB assay demonstrated that overexpression of miR-138-5p and inhibition of SIRT1 expression suppressed the expression of PI3K and p-Akt protein but had no significant effect on the expression of AKT protein. The dual-luciferase report confirmed the targeting relationship between miR-138-5p and SIRT1. The co-transfection experiment revealed that after transfecting miR-138-5p-mimics+sh-SIRT1 into SW620 and HCT116, the proliferation and invasion of cells were not different from those of the miR-NC group with unrelated transfection sequences. Conclusion: Overexpressed miR-138-5p can inhibit SIRT1-mediated PI3K/AKT signaling pathway and suppress the proliferation and invasion of CC cells, which is a promising therapeutic target for CC.

Keywords: SIRT1, PI3K/AKT signaling pathway, colon cancer, biological function

Introduction

Colon cancer (CC), as a digestive tract tumor, has a variety of predisposing factors, among which, smoking, eating habits, and poor daily routine may be risk factors for poor prognosis [1]. According to statistics, CC is the fourth most common cancer in the United States. In figure, there were about 97,220 new cases and about 50,630 deaths in 2018 [2, 3]. Although the morbidity and mortality of CC have declined in the United States, people elsewhere are still at high risk of suffering from or dying from CC [4]. The treatment decision of this disease depends largely on clinical stage and tumor metastasis. For early or non-metastatic CC, colectomy and adjuvant chemotherapy are recommended, while for those with advanced or metastatic CC, chemotherapy and immunotherapy are available [5]. Despite the continuous optimization of the treatment scheme of CC, there is still a risk of metastasis or recurrence after the treatment of CC patients [6]. Another challenge of CC is its delayed diagnosis, that is, most CC patients are already in the middle and late stage when diagnosed, losing the optimal timing for intervention and treatment [7]. Therefore, the study of high-sensitivity indicators for early diagnosis of CC patients is of great value for the treatment of CC patients and the reduction of mortality.

MicroRNAs (miRNAs) are non-coding small RNA families involved in normal cells and pathologi-
Action mechanism of miR-138-5p, SIRT1 and PI3K/AKT

cal processes. Their multi-target properties enable them to regulate a variety of oncogenes or tumor suppressor genes, and can also serve as clinical indicators for tumor diagnosis and prognosis [8]. Apart from that, miRNAs are also widely involved in the pathogenesis of human cancers, like breast cancer, lung cancer and colon cancer, as oncogenic or oncosuppressive genes [9-11]. Among them, miR-138-5p is a miRNA with abnormal expression in a variety of cancer cells. In most cases, it acts as a suppressor gene of human cancer to suppress the malignant growth, invasion and migration of cancer cells by targeting specific targets. While rarely, it functions as an oncogene of brain tumor cells or chemotherapy-resistant cells to promote tumor function [12, 13]. Although miR-138-5p is widely implicated in a variety of tumors, its specific action mechanism in CC has not been elaborated. NAD-dependent protein deacetylase sirtuin-1 (SIRT1) is a silencing regulatory protein that mediates metabolism, inflammation and DNA repair in mammals. Besides that, it can also inhibit malignant biological functions of hepatocellular carcinoma through PI3K/Akt signaling pathway [14]. As to the latter, it is a classical signaling pathway in tumor pathogenesis, which can play a tumor suppressing or carcinogenic role in tumor microenvironment, and it has some certain influence on tumor cell proliferation and apoptosis [15, 16]. However, like miR-138-5p, the pathogenesis of SIRT1-mediated PI3K/AKT signaling pathway in CC has not been elucidated.

In current study, we found that SIRT1 was a potential target site for miR-138-5p through an online target gene prediction software (http://www.targetscan.org/vert_72/). Therefore, we hypothesized that miR-138-5p could affect the biological function of CC cells by targeting SIRT1-mediated PI3K/AKT signaling pathway. In this regard, we conducted the following research.

Materials and methods

Clinical data

Totally 107 CC patients admitted to People’s Hospital of Xintai City from April 2015 to August 2018 were enrolled as CC group, including 44 male patients and 63 female patients, with an average age of 57.05±8.34 years old. In addition, 80 concurrent healthy subjects were selected as the normal group, including 32 male subjects and 48 female subjects, averagely aged 58.7±7.94 years old. No significant difference was observed in gender, age, etc. between the two groups (P>0.05), so they were comparable. Inclusion criteria: Patients diagnosed as CC by pathology or laboratory indicators [17], who met the Dukes staging criteria [18] and had not taken drugs that affects the relevant indicators in this study within the past six months. Exclusion criteria: Patients with other malignancies, who had received any treatment prior to this study, or those with infectious or blood system disorders. Written informed consent was obtained from all study subjects or their families with their full understanding of this study. This study had been approved by the Medical Ethics Committee of People’s Hospital of Xintai City.

Experimental reagents and materials

Human CC cell line SW480, SW620, HT29, HCT116 and normal colon FHC cells (Shanghai Caiyou Industries Co., Ltd., China, YS284C, YS285C, YS150C, YS2649C, YS1212C), Trans-Script Green miRNA Two-Step qRT-PCR SuperMix (Beijing Qiwei Yicheng Technology Co., Ltd., China, abx098036), TransScript II Green Two-Step qRT-PCR SuperMix (Beijing TransGene Biotechnology Co., Ltd., China, AQ301-01), MTT Kit and RIPA (Shanghai Yubo Biotechnology Co., Ltd., China, YB111105-500, YB20101-ES60), Trizol reagent (Shanghai Yiji Industriesl Co., Ltd., China, YJ58182), dual luciferase reporter gene assay kit and goat anti-rabbit IgG secondary antibody (Shanghai Qunji Biotechnology Co., Ltd., China, KA3784, MAB19-500), PBS and fetal bovine serum (FBS) (Shanghai Kemin Biotechnology Co., Ltd., China, DXT-130-070-525, DXT-10099141), BCA protein kit and ECL developer (Shanghai Yuanye Biotechnology Co., Ltd., China, R21250, R213-13), SIRT1, PI3K, AKT, p-Akt and β-Actin antibodies (Shanghai Hengfei Biotechnology Co., Ltd., China), PCR instrument (Hepeng (Shanghai) Biotechnology Co., Ltd., China, HPBIO-JX22). Shanghai Daixuan Biotechnology Co., Ltd was in charge of the design and synthesis of all primers.

Cell culture, passage and transfection

The CC cell lines were cultured in DMEM containing 10% PBS at 37°C and 5% CO₂. Trypsin
(25%) was added for complete digestion when cell adherent growth fusion reached 85%. After that, the medium was further cultured until the completion of passage. Then, the transfection. MiR-138-5p-mimics (overexpression sequence), miR negative control (miR-NC), targeted inhibition of SIRT1 RNA (si-SIRT1), targeted overexpression of SIRT1 RNA (sh-SIRT1), negative control RNA (si-NC) were transfected into cells by Lipofectamine™ 2000 kit in line with the kit instructions.

Detection methods

**QRT-PCR detection:** The total RNA was extracted from serum and cells using TRIzol kit, whose concentration and purity were then detected by UV spectrophotometer. After that, 5 μg of the obtained total RNA from serum and cells was taken respectively for cDNA reverse transcription according to the kit instructions. After reverse transcription, 1 μL of the synthesized cDNA was amplified. MiR-138-5p amplification system: cDNA: 1 μL, upstream and downstream primers: 0.4 μL each, 2X TransScript® Tip Green qPCR SuperMix: 10 μL, Passive Reference Dye (50×): 0.4 μL, and finally added nuclease-free water to 20 μL. Amplification conditions (40 cycles): pre-denaturation: 94°C for 30 s, denaturation: 94°C for 5 s, annealing: 60°C for 30 s. SIRT1 amplification system: cDNA: 1 μL, upstream and downstream primers: 0.4 μL each, 2X TransScript® Tip Green qPCR SuperMix: 10 μL, Passive Reference Dye (50×): 0.4 μL, and finally added nuclease-free water to 20 μL. Amplification conditions (40 cycles): pre-denaturation: 95°C for 30 s, denaturation: 95°C for 10 s, annealing: 60°C for 30 s. Three replicate wells were set for each sample and the experiment was conducted a total of three times. At last, 2-ΔΔct was employed for data analysis, with U6 as the internal reference of miR-138-5p, and β-actin as that of SIRT1.

**Western blot detection:** The cultured cells in each group were added with RIPA lysate to extract the total protein, and the protein concentration was determined by BCA method. Then the protein concentration was adjusted to 4 μg/μL, separated by 12% SDS-PAGE, and transferred to PVDF membrane before using 5% skim milk powder to block the PVDF membrane for 2 h. And then, SIRT1 (1:500), β-actin primary antibody (1:1000), PI3K (1:500), AKT (1:500), and p-Akt (1:500) were added and blocked at 4°C for a night. After that, rinsed the primary antibody and added with horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:1000), incubated at 37°C for 1 h, followed by a triple rinse with PBS, 5 min a time. Developed it in the dark before blotting the spare liquid on the film with a filter paper, and then illuminated by ECL to develop. Finally, the protein bands were scanned and the gray value was analyzed using Quantity One software: the relative expression level of the protein = the gray value of the target protein band/the gray value of the β-actin protein band.

**MTT assay for cell viability:** CC cells transfected for 24 h were collected, adjusted to 5×10^3 cells/well, planted in 96-well plates, and incu-
bated at 37°C for 24, 48, and 72 h. At each time point, 20 μL of MTT solution (5 μmg/mL) was added into the plates and re-cultured at 37°C for 4 h. Followed by the addition of 200 μL of dimethyl sulfoxide into each well. Finally, at 490 nm, the OD value of each group was measured by a spectrophotometer.

Transwell detection: Cells were collected 24 hours after transfection, adjusted to 3×10^4 cells/well, and inoculated in 24-well plates. After digestion with trypsin, the cells were transferred to the upper chamber, where 200 μL RPMI1640 medium was added, while the lower one was added with 500 μL RPMI1640 (containing 10% FBS), and then cultured at 37°C for 48 h. The matrix and cells of the upper chamber that did not pass through the membrane surface were scrubbed, rinsed with PBS for 3 times, and fixed with polyformaldehyde for 10 min. After that a triple flush was carried out with double distilled water before drying and staining with 0.5% crystal violet. Finally, cell invasion was observed under a microscope.

Statistical analysis

In this study, the collected data was analyzed using SPSS20.0, and the picture rendering was performed by GraphPad 7. An independent t-test was employed for inter-group comparison, and one-way ANOVA was adopted for multi-group comparison (expressed as F). Post pairwise comparison was performed by LSD-t, and repeated measurement ANOVA was applied for multi-time expression (expressed as F). Bonferroni was utilized for post-hoc test, and the correlation between miR-138-5p and SIRT1 in CC was performed by Pearson. A statistically significant difference was assumed at P<0.05.

Results

Expression and clinical value of miR-138-5p and SIRT1 in serum of CC patients

The serum miR-138-5p expression was found to be notably lower in the CC group, while on the contrary, the serum SIRT1 expression was markedly higher, with statistically significant difference (P<0.05). Pearson correlation analysis exhibited that these two were negatively correlated (P<0.05). The AUC of miR-138-5p and SIRT1 in diagnosing CC was 0.916, 0.909 respectively by plotting the ROC curve. Further analysis of the relationship between the two and the pathological data of the patients revealed that miR-138-5p and SIRT1 were closely related to Dukes staging, tumor diameter, lymph node metastasis and differentiation degree (P<0.05) (Figure 1; Table 1).

Table 1. Correlation between SIRT1 and pathological data of CC patients [n (%), mean ± SD]

<table>
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<tr>
<th>Factors</th>
<th>n=107</th>
<th>miR-138-5p</th>
<th>P value</th>
<th>SIRT1</th>
<th>t/F value</th>
<th>P value</th>
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<td>44</td>
<td>0.58±0.58</td>
<td>1.840</td>
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<td>2.07±0.07i</td>
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<td>Age</td>
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<td>0.088</td>
<td>0.691</td>
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<td>&lt;55</td>
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<td>Dukes staging</td>
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<td>3.533</td>
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<td>A.B</td>
<td>45</td>
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<td>C.D</td>
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<td>≥5 cm</td>
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<td>Degree of pathological differentiation</td>
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<td>&lt;0.001</td>
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<td>Moderate and high differentiation</td>
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<td>1.89±0.89e</td>
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Effects of miR-138-5p on proliferation and invasion of CC cells

We found that the expression of miR-138-5p was significantly lower in SW480, SW620, HT29 and HCT116 (P<0.05). After transfection of miR-138-5p-mics and miR-NC in SW620 and HCT116, the expression of miR-138-5p in the cells transfected with miR-138-5p-mics was significantly increased (P<0.05). The detection of biological function revealed that the proliferation and invasion ability of cells transfected with miR-138-5p-mimics were remarkably suppressed, and the relative expression levels of PI3K and p-Akt protein were remarkably dropped (P<0.05), while the expression of Akt was not significantly different (P>0.05) (Figure 2).

Effects of SIRT1 on proliferation and invasion of CC cells

The SIRT1 expression was markedly higher in human CC cell lines SW480, SW620, HT29 and HCT116 (P<0.05). After transfection of si-SIRT1 and si-NC in SW620 and HCT116, the expression of SIRT1 in cells transfected with si-SIRT1 was significantly inhibited (P<0.05). The cell biological functions of the two groups showed that the proliferation and invasion ability of cells transfected with si-SIRT1 were significantly inhibited and the relative expression levels of...
PI3K and p-Akt were markedly reduced (P<0.05), while the expression of Akt was not significantly different (Figure 3).

Identification of miR-5 target genes

We found the presence of targeted binding sites between SIRT1 and miR-138-5p through Targetscan7.2. Further, double luciferase activity assay was carried out to find that the luciferase activity of pmirGLO-SIRT1-3'UTR Wt was significantly decreased after upregulating miR-138-5p (P<0.05), but to that of pmirGLO-SIRT1-3'UTR Mut, there was no significant effect (P>0.05). WB detection exhibited that, after transfecting with miR-138-5p-mimics, the SIRT1 protein expression in SW620 and HCT116 cells was significantly declined (P<0.05) (Figure 4).

Co-transfection experiment

After transfection of miR-138-5p-mimics+sh-SIRT1 into SW620 and HCT116 cells, there were no significant differences between miR-138-5p-mimics+sh-SIRT1 and miR-NC (P>0.05), while the proliferation and invasion ability of miR-138-5p-mimics was significantly improved compared with that of miR-NC (P<0.05). WB assay showed that the expression levels of PI3K, p-Akt and Akt protein in miR-138-5p-mimics+sh-SIRT1 transfected cells were not markedly different from those of miR-NC transfected cells. While when compared with cells transfected with miR-138-5p-mimics, the PI3K, p-Akt and Akt proteins were significantly elevated in miR-138-5p-mimics+sh-SIRT1 transfected cells, while the expression of Akt
Action mechanism of miR-138-5p, SIRT1 and PI3K/AKT

Discussion

CC is a large intestine-associated malignancy with high mortality [19]. According to reports, CC is not only the second prevalent common cancer and the fourth most fatal tumor in women, but also the third most common and deadly tumor in men [20]. The formation of CC can be traced to the APC, RAS and TP53 gene mutations in the normal colonic mucosa, resulting in transformation of colonic epithelial cells into malignant proliferating tumors [21]. Early CC patients can be temporarily cured by resection, but they are confronted with a risk of recurrence and tumor progression [22]. Therefore, studying the regulatory mechanisms of CC is of great significance for finding potential therapeutic targets for CC patients.

More and more scholars have conducted in-depth studies on the carcinogenic or anticancer mechanism of miRNA in CC. For example, in the study of Sakaguchi [23], miR-107, as the anticancer gene of CC, could inhibit the malignant growth of CC cells by targeted inhibition of transferrin receptor 1. In another study, Islam [24] reported that, miR-142-5p, as an oncogene of CC, could promote the malignant biological behavior of CC cells by negatively regulating a tumor suppressor. There were also studies demonstrating that miR-138-5p was one of the 16 miRNAs with dysregulated expression in the body of CC patients, which may be helpful for prognosis prediction and treatment of CC patients [25]. However, the very potential regulatory role of miR-138-5p in CC has not been elaborated, and we hereby conducted the current study. The present study found that the serum miR-138-5p was significantly downregulated in CC patients, suggesting that it might be a tumor inhibitor for CC patients. Our analysis of the clinical significance of miR-138-5p revealed that the low expression of miR-138-5p was significantly correlated with the Dukes staging, tumor diameter, pathological differentiation degree and lymph node metastasis of CC patients. And by plotting the ROC curve, it was found that the AUC of miR-138-5p was >0.9, indicating that miR-138-5p enjoyed a high diagnostic value for CC patients and might be implicated in the pathological process of CC. Therefore, we further carried out cell experiments.

By comparing with FHC, we confirmed that the expression of miR-138-5p was significantly reduced in CC cell lines. Subsequently, the upregulation of miR-138-5p in SW620 and HCT116 cells showed that the biological functions of miR-138-5p-mimics transfected cells were significantly inhibited in terms of proliferation and invasion of malignant cells, and vice versa, the proliferation and invasion of CC cells were inhibited by miR-138-5p overexpression. However, how it affects the biological function of CC cells remains poorly understood. In addition, we found that SIRT1 had binding targets with miR-138-5p through online target gene prediction website. SIRT1 is a potential therapeutic target of CC with dual effects of anti-proliferation and anti-apoptosis, whose silencing is also conducive to improving chemotherapeutic sensitivity of CC patients [26, 27]. Our study
results indicated that SIRT1 was highly expressed in serum of CC patients. Meanwhile, ROC curve analysis exhibited that SIRT1 was also of high diagnostic value with a AUD of >0.9, and it was also closely related to Dukes staging, tumor diameter, lymph node metastasis and pathological differentiation, suggesting that SIRT1 might also be a potential diagnostic marker of CC. What’s more, by regulating SIRT1 expression in SW620 and HCT116, we found that SIRT1 knockdown could suppress the proliferation and invasion of CC cells. And co-transfection experiments indicated that compared with miR-138-5p-mimics, the proliferation and invasion abilities of SW620 and HCT116 were remarkably enhanced after co-overexpressing miR-138-5p and SIRT1, indicating a close correlation between miR-138-5p and SIRT1. We further verified the relationship between the two through the dual-luciferase report, which demonstrated that the overexpression of miR-138-5p markedly reduced the luciferase activity of prnGLO-SIRT1-3'UTR Wt, but had no significant effect on that of pmirGLO -SIRT1-3'UTR Mut. Moreover, SIRT1 protein expression was remarkably decreased after transfection of miR-138-5p-mimics, suggesting a targeted regulatory correlation between the two. Through the above experiments, we preliminarily proved that up-regulated miR-138-5p could inhibit SIRT1, thereby affecting the biological function of CC cells. However, the exact pathway through which it is regulated remains unclear.

Studies have shown that PI3K/AKT signaling pathway is one of the major signaling pathways involved in the regulation of human diseases, which can be activated by SIRT1 targeting and is related to the malignant progression of tumor cells [28, 29]. For instance, the study conducted by Lv [30] demonstrated that SIRT1 could promote malignant progression of endometrial cancer by activating PI3K/AKT/GSK-3β signaling. Here, the detection of PI3K/AKT signaling pathway related proteins in CC cells with the overexpressed miR-138-5p or under-expressed SIRT1 protein revealed that, PI3K and p-Akt were significantly declined in SW620 and HCT116 with upregulation of miR-138-5p or silencing of SIRT1 protein, while the expression of Akt was not significantly different, suggesting that miR-138-5p might inhibit the activation of PI3K/Akt signaling pathway by regulating SIRT1 to promote p-Akt phosphorylation. Ac-
According to previous studies, the activation of the PI3K/AKT signaling pathway can promote the proliferation and invasion of CC cells, and it is observed that its inhibition in the CC mouse model can prevent tumor progression and increase the apoptosis rate of cancer cells [31, 32].

In conclusion, miR-138-5p affects the proliferation and invasion of CC cells by targeting SIRT1-mediated PI3K/AKT signaling pathway, which is a promising diagnostic tool and therapeutic target for CC in clinical practice. However, there is still room for improvement in this study. First, we can supplement the follow-up of CC patients to further analyze the risk factors affecting the survival rate of patients. Second, we can discuss the predictive value of miR-138-5p and SIRT1 on the prognosis of CC patients. In the future, we will definitely conduct supplementary research as described above.

Disclosure of conflict of interest

None.

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