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Original Article

Effect of antisense microRNA targeting survivin on rectal cancer HRC-9698 cells and its mechanism

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Abstract: Background: Rectal cancer seriously threats to human health. Traditional chemotherapy drugs might kill rectal cancer cells while easy cause side effects and clinical complications. Therefore, it is necessary to explore possible new methods for rectal cancer treatment. Survivin is an important tumor-specific protein. Previous researches showed it may be closely related to nasopharyngeal carcinoma. Its role in rectal cancer remains unclear. Methods: Cultivate human rectal cancer HRC-9698 cells. Antisense microRNA targeting survivin and control miRNA were constructed and transfected to HRC-9698 cells. MTT assay was applied to detect cell growth. Flow cytometry was used to test cell apoptosis. Western blot was performed to detect Osteopontin expression level. Colony formation and transwell assay were used to test cell clone formation and invasion abilities. Results: Antisense microRNA targeting survivin can inhibit HRC-9698 cell proliferation and induce its apoptosis. Antisense microRNA targeting survivin decreased osteopontin expression level. Overexpressed osteopontin inhibited antisense microRNA targeting survivin induced cell apoptosis. Antisense microRNA targeting survivin suppressed HRC-9698 cells' colony formation and invasion abilities. Conclusion: Antisense microRNA targeting survivin induced osteopontin participated HRC-9698 cell apoptosis. Antisense microRNA targeting survivin inhibited HRC-9698 cell colony formation and invasive abilities, indicating that survivin may play its anti-tumor effect through inducing cell apoptosis and inhibiting cell metastasis and invasion.

Keywords: miRNA, HRC-9698, proliferation, apoptosis, survivin, osteopontin

Introduction

Rectal cancer is a common malignant tumor of digestive system, which refers to the location from the rectum of the sigmoid colon to the dentate line. The incidence of the disease is about 4.2%, and the onset age is about 40 [1-3]. At present, it is thought that the occurrence of rectal cancer has a close relationship with diet. High protein and fat, and low fiber in the diet is one of the main reasons inducing rectal cancer [4]. Following the growth in the living standard, the incidence of rectal cancer is on the rise, and the onset age becomes smaller [4]. As a result, the disease is a serious threat to human health and quality of life.

Traditional treatment methods mainly include surgery, chemotherapy and radiotherapy. There are many defects and clinical side effects in these traditional treatments. Specially, chemotherapy drugs may easy cause side effects and clinical complications when killing the rectal cancer cells [5-8]. Therefore, it is necessary to explore possible new treatment methods for rectal cancer. With the development of science and technology, rectal cancer is likely to be combined treated from the gene, protein, and even molecular level [9, 10].

Survivin is an important tumor specific protein that plays an important role in the process of cell death attracts many attentions in recent years [11-15]. Survivin can regulate the process of cell cycle [13]. Studies hypothesized that survivin may play a regulation role in the process of cell apoptosis. However, whether survivin involved in rectal cancer cell apoptosis directly was still lack of report. More importantly, the existing researches suggested that it may be closely related to the incidence of nasopharyngeal carcinoma, survivin may be involved in

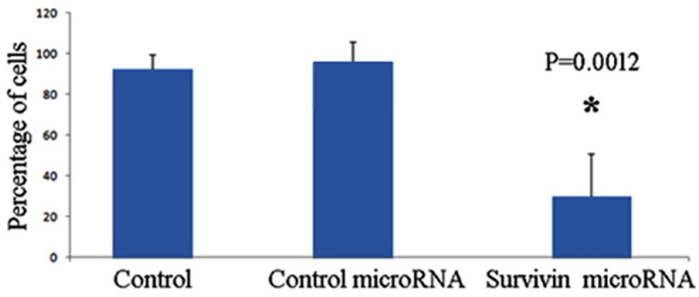

Figure 1. Antisense miRNA for survivin inhibited HRC-9698 cells proliferation.

cancer cell invasion and metastasis [16-20]. At present, the molecular mechanism of survivin in nasopharyngeal carcinoma occurrence, invasion and metastasis was still lack of investigation. Whether survivin participates in rectal cancer cell invasion and metastasis is still unclear. This article aimed to explore these two problems.

Osteopontin is a non-collagen glycoprotein that widely concerned in recent years. Tumor cells may perform its invasion and metastasis function through the synthesis and secretion of osteopontin [18]. Research suggested [18] that survivin may regulate tumor metastasis by affecting osteopontin levels. This article tried to investigate the possible relationship between survivin and osteopontin in rectal cancer.

This article selects human rectal cancer HRC-9698 cell as the experimental material. Antisense miRNA of survivin and its control miRNA was transfected to human rectal cancer HRC-9698 cells. Flow cytometry and western blot were applied to investigate the effect and molecular mechanism of survivin to HRC-9698 cell, so as to provide information for the treatment of rectal cancer.

Materials and methods

Cell and reagents

Human rectal cancer HRC-9698 cells were purchased from Shanghai Bioleaf biological technology co., LTD. MTT reagent was bought from the Beijing Dingguo biotechnology co., LTD. Apoptosis detection reagents including FITC-Annexin-V and caspase 3 activity detection kits were got from Beyotime Biotechnology. Anti-survivin and anti-osteopontin primary antibodies were bought from Sigma. RPMI-1640 medium and fetal bovine serum were purchased from Shanghai Fengshou biological technology co., LTD. Antisense miRNA for survivin and control miRNA were provided by Suzhou Genepharma biological co., LTD. Liposome transfection reagent was got from Dingguo biotechnology co., LTD.

Human rectal cancer HRC-9698 cell culture

HRC-9698 cells were maintained in RPMI-1640 medium supplemented with 20% fetal calf serum in a humid atmosphere containing 5% CO_2 at 37°C as a matter of routine [21].

Cell transfection

HRC-9698 cells were digested by 0.1 nM trypsin and 0.01% EDTA at 37°C for 10 min. After added fresh medium, the cells were centrifuged at 1000 rpm/min for 5 min on the day before transfection. When the cell density achieved 95%, antisense miRNA for survivin and control miRNA were transfected to the cell through liposome transfection method. 24 h after transfection, the cells were changed back to the fresh medium. MTT assay was applied at 36 h after transfection.

MTT detection

MTT assay was applied to detect HRC-9698 cells proliferation after transfection as a matter of routine [21].

Flow cytometry

Flow cytometry was used to detect apoptosis of the transfected HRC-9698 cells as a matter of routine [21].

Western blot

Western blot was used to detect survivin and osteopontin expression level in transfected HRC-9698 cells as a matter of routine [21].

Caspase-3 activity detection

Caspase-3 activity detection kit was applied to detect caspase-3 activity according to the manual.

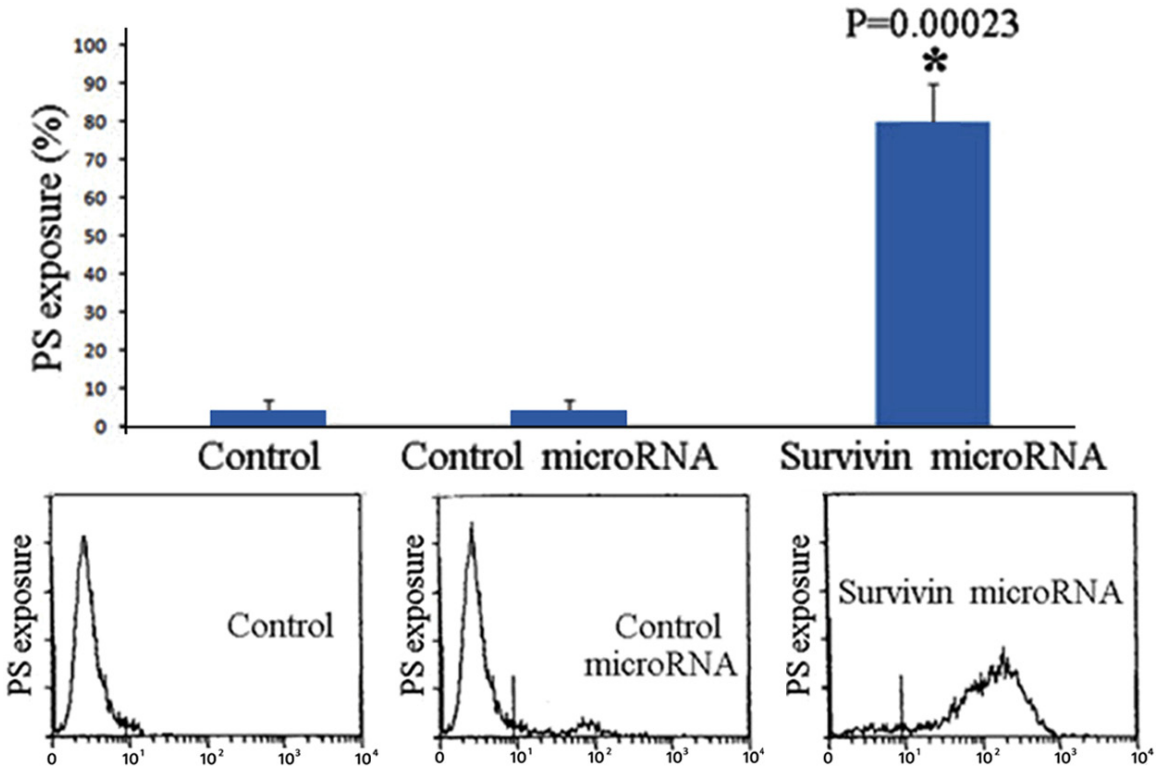

Figure 2. Antisense miRNA for survivin induced HRC-9698 cells PS exposure.

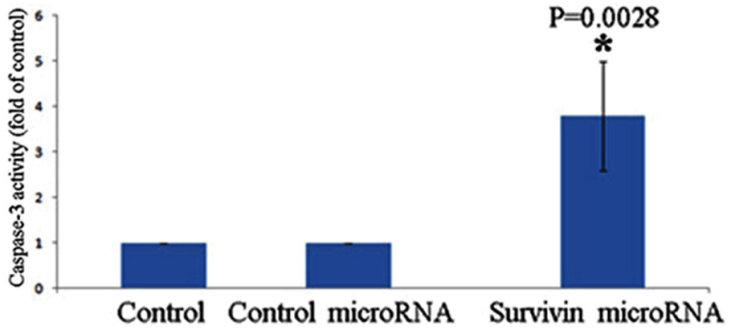

Figure 3. Antisense miRNA for survivin induced HRC-9698 cells caspase-3 activation.

Colony formation assays

Cells were plated on soft agar and cultured for 2 weeks. The colonies were stained with Giemsa for 30 min after fixation with 10% formaldehyde for 15 min as a matter of routine [22]. The colony number in each well was counted.

Transwell assay

Solid medium was but on the upper chamber of the Transwell plate, and medium containing chemotactic factor was laid on the lower chamber. The transfected cells were seeded on the upper chamber as a matter of routine [22]. The number of cells in the lower chamber after 48 h cultivation was counted.

Statistical analysis

All statistical analyses were performed using SPSS13.0 software (Chicago, IL). Numerical data were presented as means and standard deviation (± SD). Differences between means were analyzed using one-way ANOVA. $P<0.05$ was considered with statistical significant difference, and $P<0.01$ was considered with high significant difference.

Results

Antisense miRNA for survivin inhibited HRC-9698 cells proliferation

To study the effect of survivin on human rectal cancer HRC-9698 cells, we constructed antisense miRNA for survivin and its control. Cell proliferation was determined by MTT assay. As shown in **Figure 1**, compared with the control,

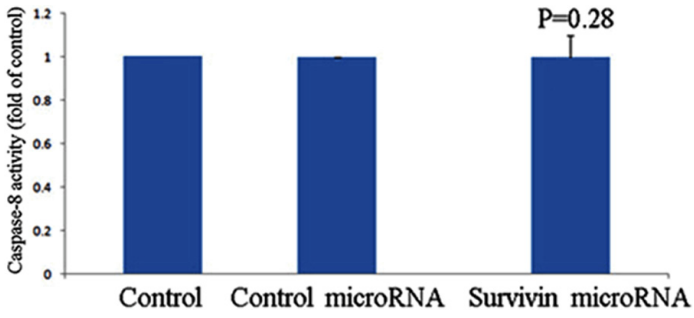

Figure 4. Antisense miRNA for surviving failed to activate caspase-8. It was reported [18] that survivin may regulate cancer metastasis through impacting osteopontin expression level; we tried to investigate the relationship between survivin and osteopontin in rectal cancer. Our study also explored the role of osteopontin in antisense miRNA for surviving induced apoptosis. Osteopontin expression level decreased significantly after HRC-9698 transfected with antisense miRNA for survivin (**Figure 5**). Caspase-3 activity reduced obviously in the cell overexpressed osteopontin (**Figure 6**). It revealed that antisense miRNA for survivin induced osteopontin mediated cell apoptosis.

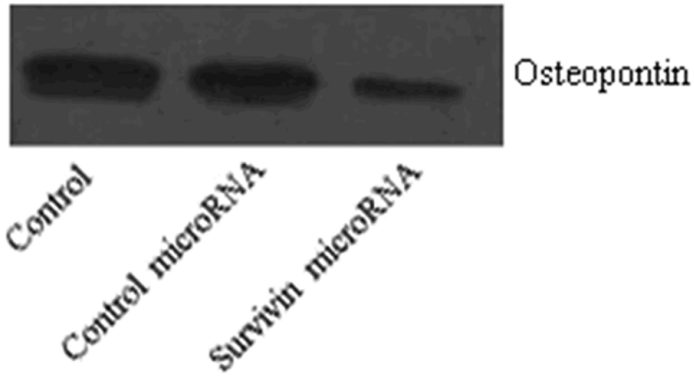

Figure 5. Antisense miRNA for survivin decreased osteopontin level.

cells transfected with antisense miRNA for survivin grow slower obviously. It suggested that antisense miRNA for survivin inhibited HRC-9698 cells proliferation.

Antisense miRNA for survivin induced HRC-9698 cells apoptosis

To further explore the regulating role of survivin on HRC-9698 cells, flow cytometry and caspase-3 activity detection kit were applied to detect whether antisense miRNA for survivin can induce HRC-9698 cells apoptosis. As shown in **Figures 2** and **3**, antisense miRNA for survivin significantly induced HRC-9698 cells PS exposure and caspase-3 activation, which suggested that antisense miRNA for survivin induced HRC-9698 cells apoptosis.

Antisense miRNA for survivin down-regulated osteopontin level

To further investigate the mechanism of survivin on inducing HRC-9698 cells apoptosis. It was found that caspase-8 was not activated (**Figure 4**) during the process of antisense miRNA for survivin induced HRC-9698 cells apoptosis. While caspase-3 was activated. It suggested that antisense miRNA for survivin induced HRC-9698 cells apoptosis was mainly through the mitochondrial pathway instead of the death receptor mediated external apoptosis pathway.

Antisense miRNA for survivin inhibited HRC-9698 colony formation

To further explore the role of survivin on rectal cancer HRC-9698 cell, we studied whether antisense miRNA for survivin affect clone formation ability of HRC-9698 cell. As shown in **Figure 7**, antisense miRNA for survivin inhibited HRC-9698 cell clone formation ability.

Antisense miRNA for survivin inhibited HRC-9698 cell invasion

To further study the role of survivin on rectal cancer HRC-9698 cell, we investigated whether antisense miRNA for survivin affect HRC-9698 cell invasion ability. As shown in **Figure 8**, antisense miRNA for survivin inhibited HRC-9698 cell invasion ability.

Discussion

Survivin is an important protein that regulates cell death [11-15]. Studies suggested that it may be involved in the occurrence and progression of nasopharyngeal carcinoma. However, there is still lack of reports about whether survivin is involved in rectal cancer. Therefore, based on human rectal cancer HRC-9698 cells model, we investigate the regulating role of survivin on rectal cancer HRC-9698 cell by adopting antisense miRNA for survivin.

Transfection effect was first analyzed. The results showed that survivin level in rectal can-

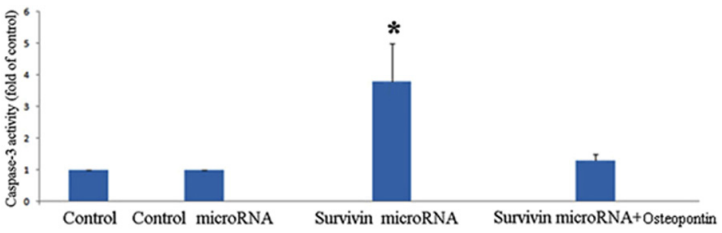

Figure 6. Overexpressed osteopontin inhibited antisense miRNA for survivin induced cell apoptosis.

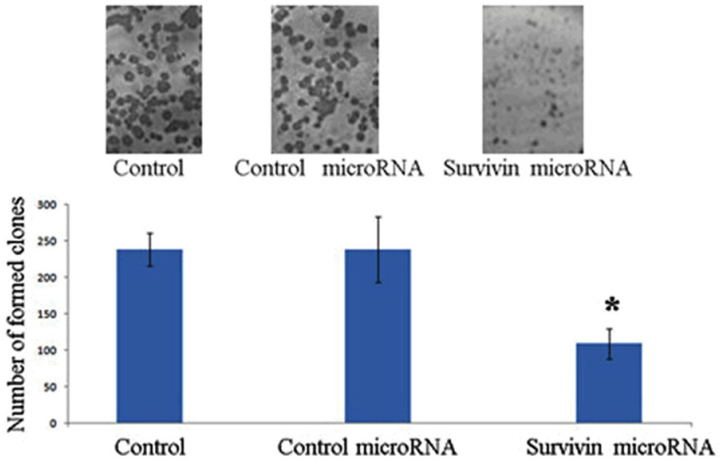

Figure 7. Antisense miRNA for survivin inhibited HRC-9698 colony formation ability.

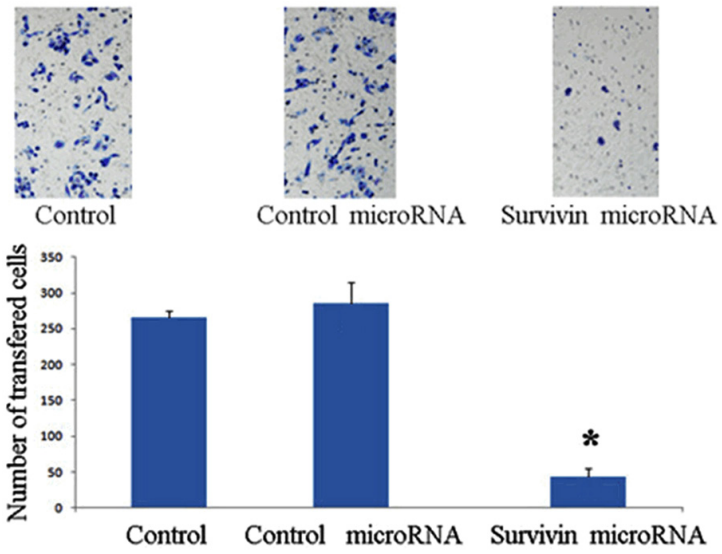

Figure 8. Antisense miRNA for survivin inhibited HRC-9698 cell invasion ability.

cer HRC-9698 cell decreased significantly after transfection for 36 hours. MTT exhibited that antisense miRNA for survivin obviously inhibited HRC-9698 proliferation. Research suggested that [18] survivin may regulate tumor metastasis through affecting osteopontin level. We explored the possible relationship between survivin and osteopontin in rectal cancer. Antisense miRNA for survivin could induce cell apoptosis proved by PS exposure and caspase-3 activation.

There are two major cell apoptosis signaling pathways, namely the mitochondria mediated internal signaling pathway and death receptor mediated external signaling pathway. This paper also investigates the HRC-9698 cell apoptosis signaling pathway induced by antisense miRNA for survivin. A main characteristic of death receptor mediated external signaling pathway is caspase-8 activation. We failed to find caspase-8 activation in antisense miRNA for survivin inducing HRC-9698 cell apoptosis, while caspase-3 was activated under the same condition. Caspase-3 activation is the common characteristics in mitochondrial mediated internal signaling pathway and death receptor mediated external signaling pathway. For we eliminated death receptor mediated external signaling pathways, it can be considered that antisense miRNA for survivin induced HRC-9698 cell apoptosis through mitochondrial mediated internal signaling pathway. This is constant with the previous result in nasopharyngeal carcinoma [16-20].

Osteopontin is a type of secretion protein that still lack of information in cancer cell. Specially, antisense miRNA for survivin can decrease osteopontin level. To investigate the relationship between osteopontin and survivin in cell regulation, we overexpressed osteopontin at the same time with antisense miRNA for survivin. Constant with previous result, overex-

pressed osteopontin suppressed antisense miRNA for survivin induced cell apoptosis [16-20].

Malignant proliferation and invasive ability are important characteristic for tumor, and also challenge in current clinical cancer treatment. The effect of secretion protein osteopontin in antisense miRNA for survivin induced HRC-9698 cell apoptosis urge us to investigate the role of survivin in HRC-9698 cell clone formation ability and invasive ability. It was found that antisense miRNA for survivin suppressed HRC-9698 cell clone formation ability and invasive ability, suggesting that survivin may play anti-tumor role by inducing rectal cancer cell apoptosis and inhibiting cancer migration and invasion. It still needs further investigation.

Further study should adopt rectal cancer animal model (for example, rat model) and inject HRC-9698 cells transfected with antisense miRNA for survivin to test target surviving effect.

There are still some parts of our study to improve. Firstly, animal model should be adopt for further confirming survivin effect; secondly, survivin expression level should be detected in animal model at different stages; thirdly, clinical specimens should be collected to test the animal experiments result.

In short, our study revealed that antisense miRNA for survivin induced osteopontin mediated rectal cancer HRC-9698 cell apoptosis. Antisense miRNA for survivin inhibited rectal cancer HRC-9698 cell clone formation ability and invasive ability, suggesting that survivin might play anti-tumor role by inducing rectal cancer cell apoptosis and inhibiting cancer migration and invasion. Our results provide new target and theory basis for rectal cancer treatment.

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Disclosure of conflict of interest

None.

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