MicroRNA-873 inhibits proliferation and induces apoptosis by targeting CXCL1 in gastric cancer

Xuyan Chen, Renpin Chen, Wei Wu, Zhiming Huang

Department of Gastroenterology and Hepatology, The First Affiliated Hospital of Wenzhou Medical University, Zhejiang, P. R. China

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Abstract: MiRNAs can be considered as a proto-oncogene and a tumor suppressor gene by the targeted inhibition of mRNAs translation. More and more studies show that miR-873 can inhibit the growth of tumor. In spite of this, the mechanism by which miR-873 inhibits the development of gastric cancer remains unclear. Chemokine (C-X-C motif) ligand 1 (CXCL1) is a secreted growth factor that signals through the G-protein coupled receptor. CXCL1 plays a role in inflammation, and aberrant expression of this protein is associated with the growth and progression of certain tumors. In order to explore the relationship between miR-873 and the development of gastric cancer, we analyzed the expression level of miR-873 in 72 cases of gastric cancer, the prognosis and survival rate. What’s more, we tested the effect of miR-873 on the proliferation and apoptosis of gastric cancer cells. Through the bioinformatics analysis, the action target of miR-873 was predicted and verified. The results showed that miR-873 was significantly down-regulated in gastric cancer tissue and gastric cancer cell line. Clinical data showed that the low expression of miR-873 was significantly associated with poor prognostic features of GC (including serum alpha fetal protein (AFP) level), tumor size and TNM stage. Gain-of-function and loss-of-function studies showed that miR-873 can inhibit the proliferation of HGC27 cells and induce apoptosis of HGC27 cells. Bioinformatics analysis showed that the 3'UTR region of CXCL1 mRNA contains the binding site of miR-873. CXCL1 knockdown abrogated the effects of miR-873 deletion on GC cells with decreased cell proliferation and increased apoptosis. Taken together, our results reveal that miR-873 can be used as a tumor suppressor that prevent proliferation and induce apoptosis in GC by targeting CXCL1.

Keywords: MiR-873, gastric cancer, apoptosis, prognosis, CXCL1

Introduction

Gastric cancer is the fourth most common malignant tumor in the world, and its incidence and mortality rate is very high. According to statistics in the newly-diagnosed cancer, the gastric cancer accounts for about 10% and it is the second major cause of cancer death [1]. In recent decades, with the continuous improvement of medical level and the progress of surgery and chemotherapy technology, cancer prevention and cure has been effectively controlled [2]. However, as the most common malignant tumor, early diagnosis is still a major obstacle to the successful treatment of gastric cancer [3]. Therefore, exploring a new biomarker for gastric cancer may provide a potential target for early diagnosis and treatment. Previous studies have indicated that non-coding RNA might be involved in the occurrence and development of a variety of diseases, which provides a new perspective for us to explore new targets for prevention and cure of gastric cancer.

MiRNA is a single stranded non-coding RNA containing 17-25 nucleotides, which was originally thought to be the noise generated during the transcription process [4]. Later studies showed that miRNA can regulate multiple physiological processes by specific binding with the target point 3'UTRs and inhibiting the translation of the target in the post transcriptional levels [5-7]. More and more studies have showed that miRNA can regulate cell proliferation, apoptosis and differentiation, which can be considered as a proto-oncogene and a tumor suppressor gene as well, and it plays an impor-
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Therefore, the identification of tumor-associated miRNAs and exploration of the mechanisms of miRNAs in the development of cancer become an important issue of cancer research. The study showed that inhibiting the expression of miR-873 can promote the metastasis of breast cancer [11]. In contrast, over-expression of miR-873 can inhibit the invasion and proliferation of cancer cells, change the cell cycle progression of breast cancer, and induce the apoptosis of breast cancer cells [12]. In fact, miR-873 plays an important role in the occurrence and development of various cancers. However, the role of miR-873 in the development of gastric cancer is still not very clear.

In this article, in order to clarify the role and mechanism of miR-873 in the pathogenesis of gastric cancer, we analyzed the effects of miR-873 on the proliferation and apoptosis of gastric cancer cells. Through the bioinformatics method, the action target of miR-873 was predicted, and it was verified by the dual luciferase reporting system. The target of miR-873 was knocked out by RNA interference technology to verify whether miR-873 can regulate the proliferation and apoptosis of gastric cancer cells through inhibiting its action target. Explore the impact of miR-873 in the mechanism of apoptosis of gastric cancer cells by detecting changes in the expression levels of apoptosis-related proteins.

**Materials and methods**

**Tissue collection**

Fresh frozen GC and adjacent tissues were obtained from 72 patients who were diagnosed with GC and underwent radical gastrectomy at the department of Gastroenterology and Hepatology, The First Affiliated Hospital of Wenzhou Medical University from January 2009 to December 2011. None of these patients underwent local or systemic therapy before surgery. This research was approved by the Research Ethics Committee of Wenzhou Medical University, China, and all patients signed the written informed consent.

**Cell culture conditions and transfection**

The human gastric cancer cell lines MGC803, SGC7901, BGC823, HGC27 and MKN45 were purchased from American Type Culture Collection (ATCC, USA), and the normal gastric mucosal epithelial cell line GES-1 was purchased from CICLR (Beijing, China). Cells were cultured in (RPMI) 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma, USA), at 37°C, in 5% CO2 incubator (Memmert, Germany). MiR-873 mimics and miR-873 inhibitors were purchased from Ribobio (Ribobio, China). Cell lines were transfected with mimics or miR-873 inhibitors with Lipofectamine 2000 (Invitrogen) following the user manual.

**Quantitative real-time PCR**

Total RNA was extracted and isolated from tissue samples or cell samples using Trizol reagent (Invitrogen, USA) following the user manual. SYBR PremixExTaq™ (Takara, Japan) was used in Quantitative real-time PCR (qRT-PCR) on BIO-RAD MY IQ (USA). All primer sequences are shown in Table 2. The relative expression of miR-873 was shown as fold difference relative to U6. The cell in which miR-873 expression is higher than that in GES-1 cells will be used for subsequent study.

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**Table 1. Correlation of the expression of miR-873 with clinicopathologic features**

<table>
<thead>
<tr>
<th>Clinicopathologic features</th>
<th>n (72)</th>
<th>Relative expression of miR-873</th>
<th>P</th>
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<tbody>
<tr>
<td>Age (year)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 50</td>
<td>32</td>
<td>14</td>
<td>18</td>
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<tr>
<td>≥ 50</td>
<td>40</td>
<td>23</td>
<td>17</td>
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<tr>
<td>Sex</td>
<td></td>
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<tr>
<td>Male</td>
<td>39</td>
<td>18</td>
<td>21</td>
</tr>
<tr>
<td>Female</td>
<td>33</td>
<td>14</td>
<td>19</td>
</tr>
<tr>
<td>Serum AFP level (ng/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 20</td>
<td>14</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>≥ 20</td>
<td>58</td>
<td>43</td>
<td>15</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 5</td>
<td>26</td>
<td>7</td>
<td>19</td>
</tr>
<tr>
<td>≥ 5</td>
<td>46</td>
<td>33</td>
<td>13</td>
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<tr>
<td>TNM tumor stage</td>
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<td></td>
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<tr>
<td>I+II</td>
<td>51</td>
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<td>33</td>
</tr>
<tr>
<td>III+IV</td>
<td>21</td>
<td>16</td>
<td>5</td>
</tr>
</tbody>
</table>

Compared with low miR-873 expression group, *P < 0.05, **P < 0.01.
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Bioinformatics methods and luciferase assay

We analyzed the action target of miR-873 in the human genome by online software, i.e. TargetScan, miRBase and PicTar. Combined with three software analysis results, the highest scoring targets were selected for subsequent experimental study. It was concluded that CXCL1 was most likely to be the action target of miR-873. Using the primers designed in Table 2, the human wild type CXCL1 3'-UTR was amplified in vitro. Perform site-directed mutagenesis of wild type CXCL1 3'-UTR with fast mutation kit provided by NEB Company. After recovery, the PCR product was cloned into the psiCHECK-2 vector downstream with luciferase encoding memory, and the effect of miR-873 on the luciferase activity was detected in 393T cells (ATCC, USA).

RNA interference targeting CXCL1

Through the preliminary experiment, we proved that CXCL1 is the action target of miR-873. However, a miR-873 often has multiple action targets. It is necessary for us to knockdown CXCL1 gene to explore whether miR-873 can affect the proliferation and apoptosis of GC cells. RNA interference technology is a conservative gene monitoring mechanism in the evolution, and it can specifically silence the target gene. CXCL1 (Gen-Bank Gene ID: 2919) gene was retrieved on the NCBI website, and the siRNA sequence of IGF-1 gene was designed by using Invitrogen (an online software). Well designed siRNA sequence is synthesized by Ribobio. CXCL1 siRNA was transfected into gastric cancer cells by Lipofectamine 2000 (Invitrogen). Western Blot was used to verify the transfection effect.

Cell proliferation assays

Cell Proliferation Reagent Kit I was used to detect the proliferation of cells that were transfected by CXCL1 siRNA. After the transfection of miR-873 mimics, miR-873 inhibitors or CXCL1 siRNA for 48 h, the cells were transferred to a 96-hole cell culture plate. Regulate the cell concentration of 5,000 cells per hole. After 24 hours, the cell proliferation was recorded and the data were analyzed according to the instructions provided by the kit. Each experiment was repeated 3 times.

Flow-cytometric analysis of apoptosis

After the transfection of miR-873 mimics, miR-873 inhibitors or CXCL1 siRNA for 48 h, collect cells by trypsin digestion. After heavy suspension, regulate the cell concentration of 105 cells/ml. Cell apoptosis rate was detected by flow cytometry after double staining of FITC and PI. According to the characteristics of the cell, the cells were divided into living cells, dead cells, early apoptotic cells and late apoptotic cells. The proportion of apoptotic cells was calculated by the flow cytometry software. All samples were tested three times.

Western blot analysis

Recovering the treated cells, RIPA lysis buffer (Beyotime, Haimen, China) was used to split the cells, and the total protein was extracted. After the protein was purified, take about 50 μg of...
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the total protein. Conduct 10% SDS-PAGE electrophoresis. After the PAGE gel was transferred to the membrane through nitrocellulose membrane, the membrane was incubated with the first antibody which was purchased from Abcam (Abcam, USA). And then the membrane was incubated with the second antibody marked HRP (Beyotime, Haimen, China) for the night. Analyze the optical density of protein bands of CXCL1, P53, Caspase3 and GAPDH by ImageJ software (National Institutes of Health, Bethesda, MD, USA) after the nitrocellulose membrane was fixed and developed.

Statistical analysis

Data are presented as mean ± SEM. A Pearson chi-squared test was applied to determine clinicopathological correlations. The Kaplan-Meier method was used to calculate the survival curves. Significance was determined by the log-rank test. The difference among groups was determined by ANOVA analysis and comparison between two groups was analyzed by the Student’s t-test using GraphPad software version 5.0 (GraphPad Software, CA). Difference were considered significant when $P < 0.05$.

Results

MiR-873 is downregulated and correlated with poor prognosis in GC

By retrospective cohort study, we used qRT-PCR to detect the expression levels of miR-873 in 74 pairs of GC tissues and paired adjacent tissues. The expression level of miR-873 in GC tissue was significantly lower than that in adjacent tissues (Figure 1A). According to the middle value of the expression level of miR-873, the research group was divided into two parts, namely high expression and low expression of miR-873. As shown in Table 1, in the GC patient population, patients with lower miR-873 expression had higher AFP (alpha-fetoprotein) levels, larger medium tumor size, and higher TNM tumor stage. In addition, in order to explore the relationship between the expression of miR-873 and the prognosis of GC, we performed Kaplan-Meier survival analysis on 74 patients. The results showed that patients with lower miR-873 expression had shorter survival time than those with higher expression of miR-873.
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Expression of miR-873 is upregulated on HGC27 cells

The expression of miR-873 was detected in MGC803, SGC7901, BGC823, HGC27 and MKN45 cells, and normal GES-1 cells with qRT-PCR. The results showed that the expression of miR-873 on HGC27 cells is higher than that in MGC803, SGC7901, BGC823 and MKN45 cells (Figure 2, P < 0.05). And the HGC27 cells can be used for subsequent in vitro experiment study.

CXCL1 was identified as a target of miR-873

In order to further explore the mechanism of miR-873 in the occurrence and development of GC, we used bioinformatics methods. Analyze the action target of miR-873 by online software, i.e. TargetScan, miRBase and PicTar. The results showed that there was a binding site in the 3'-UTR region of CXCL1 mRNA, and the CXCL1 was most likely to be an action target of miR-873 (Figure 3A). The reporting assay of dual luciferase showed that miR-873 could significantly inhibit the activity of luciferase of wild type CXCL1 3'-UTR, but it had no significant effect on the activity of luciferase with mutant CXCL1 3'-UTR (Figure 3B). The result indicates that CXCL1 is a direct action target of miR-873.

MiR-873 suppresses cell proliferation and induces cell apoptosis, and CXCL1 knockdown has the same effect as miR-873

In order to explore whether CXCL1 is a key factor for function execution of miR-873, we knocked down the expression of CXCL1 by siRNA. The results suggested that miR-873 mimics could inhibit the proliferation and of HGC27 cells and induce the apoptosis of HGC27 cells (Figure 4). However, HGC27 cells were treated with CXCL1 siRNA and miR-873 mimics, the proliferation and apoptosis of HGC27 cells were not significant. The result showed that the knockdown gene of CXCL1 has the same effect as miR-873 mimics on the proliferation and apoptosis of HGC27 cells. The effect of miR-873 on the proliferation and apoptosis of HGC27 cells was achieved through the action target of CXCL1.

MiR-873 suppresses expression of CXCL1, caspase3 and p53 in vitro

Caspase3, caspase9 and p53 are proteins closely related to cell apoptosis. In this study, in order to explore the effect of miR-873 on the proliferation and apoptosis of GC cells, we detected the expression levels of CXCL1, Caspase3 and p53 in GC cells after the treatment with miR-873 mimics or miR-873 inhibitors. The results showed that the expression levels of CXCL1, Caspase3 and p53 were sig-
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Significantly decreased after the treatment with miR-873 mimics. While after the treatment with miR-873 inhibitors, CXCL1, Caspase3 and p53 expression levels were significantly increased (Figure 5). The result indicates that miR-873 may regulate the proliferation and apoptosis of GC cells in vitro by directly acting CXCL1. At the same time, it can regulate the apoptosis of GC cells by affecting the expression of p53 and Caspase3.

Discussion

Exploration and identification for targets of the malignant tumor therapy is a very important topic in cancer research. Gastric cancer is a kind of high mortality rate of nausea with poor prognosis [13]. Although the strategy of preventing gastric cancer is more and more mature, there are still many difficulties in the early diagnosis of gastric cancer. The occurrence and development of tumor is a very complicated process [14]. Through the analysis on daily routine and diet structure, there are various factors that may induce gastric cancer, e.g. irregular work and rest, diet, and unhealthy food [15-17]. What’s more, through the
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Earlier studies have indicated that miR-873 is involved in the pathogenesis of a variety of tumors, which are down-regulated in glioblastoma, lung adenocarcinoma, and colorectal cancer [27-29]. However, the role of miR-873 in gastric cancer is still unknown. In our earlier study, we found that miR-873 was significantly down-regulated in the tumor tissues of patients with gastric cancer, suggesting that miR-873 may be involved in the development of gastric cancer. In subsequent experiments, we collected data from patients with gastric cancer. The statistical analysis suggested that the prognosis and survival rate of patients with gastric cancer were significantly correlated with the expression of miR-873 in the samples. As a result, as the research object, miR-873 is applied in the following experimental study. In the experiment, we found that the expression of miR-873 in gastric cancer was significantly lower than that in the adjacent tissues of gastric cancer. And mir-873 could inhibit the proliferation of gastric cancer cells and induce the apoptosis of gastric cancer cells. Therefore, miR-873 could be used as an important tumor suppressor in the prevention and treatment of gastric cancer. However, miRNAs needs to be mediated through its downstream target to play a role in participating in the regulation of biological physiology and molecular mechanism. Therefore, we have predicted and verified that the action target of miR-873 in gastric cancer cells was CXCL1 through bioinformatics method. CXCL1 is a secreted growth factor that signals through the G-protein coupled receptor. CXCL1 plays a role in inflammation, and aberrant expression of this protein is associated with the growth and progression of certain tumors.

However, a miRNA often has multiple action targets. In order to verify that CXCL1 is a direct action target of miRNA in the pathogenesis of gastric cancer, we knocked down CXCL1 in gastric cancer cells with siRNA. The results showed that the expression of CXCL1 was down-regulated and the effect of miR-873 on
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the proliferation and apoptosis of HGC27 cells was significantly offset, which further confirmed the relationship between miR-873 and CXCL1 in gastric cancer cells. In the experiment, we found that miR-873 not only inhibited the expression of CXCL1, but also induced the expression of caspase3 and p53 proteins related to apoptosis. However, the mechanism of inducing the expression of caspase3 and p53 by miR-873 should be further explored in the future.

In summary, our studies provides novel evidence that downregulation of miR-873 can inhibit the proliferation, and induce the apoptosis of gastric cancer by regulating the expression of CXCL1. These data suggest that miR-873 and its downstream targets may be potential therapeutic targets in human gastric cancer.

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Disclosure of conflict of interest
None.

Address correspondence to: Zhiming Huang, Department of Gastroenterology and Hepatology, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province, P. R. China. E-mail: hzhiming1234@163.com

References

[18] Bradbury KE, Appleby PN, Key TJ. Fruit, vegetable, and fiber intake in relation to cancer risk: findings from the European Prospective...
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