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MiR-873-5P controls gastric cancer progression by targeting hedgehog-GLI signaling

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Gastric cancer is one of the most common human malignancies. Thus, it is important to explore the specific mechanism in which gastric cancer is induced. The level of miR-873-5p was determined using real time PCR. The expression of Gli1 was determined using western blot and immunohistochemistry. A specific siRNA targeting Gli1 was selected. The role of Gli1 and miR-873-5p on gastric cancer cell viability, apoptosis and cell cycle was determined using MTT assay and flow cytometry, respectively. The 3'untranslated region (3'UTR) of Gli1 was cloned into the pmirGLO plasmid. Dual luciferase reporter assay was applied to determine the target gene of miR-873-5p. The expression of miR-873-5p was decreased in gastric cancer, while the expression of Gli1 was significantly enhanced. Overexpression of miR-873-5P decreased cell viability, increased cell apoptosis and cell cycle arrest. Meanwhile, knockdown of Gli1 obviously induced SGC-7901 cell apoptosis and cell cycle arrest. Dual luciferase reporter assay showed that Gli1 was the target gene of miR-873-5p. More importantly, inhibition of miR-873-5p obviously decreased the protein expression of CyclinB1 and Bcl2 even in cells transfected with si-Gli1. To conclude, MiR-873-5p functions as a tumor suppressor in gastric cancer mainly by targeting Gli1.

1. Introduction

Gastric cancer is one of the most common human malignancies (Hooper and Scott 2005; Ferlay et al. 2010) and was the third most common cancer-related mortality in 2005 in China (Yang 2006; Jing et al. 2012). Surgical resection combined with chemotherapy and radiation therapy is the major treatment method for gastric cancer (Macdonald et al. 2001). However, about 60% of the gastric cancer patients demonstrated locally advanced and metastatic disease, leading to a significantly decreased therapeutic efficacy (Torre et al. 2015). At present, increasing ocogenes and tumor suppressors associated with gastric cancer have been identified (Gabison et al. 2005). Thus, it is important to identify new treatment targets for gastric cancer.

The Hedgehog (Hh) signaling plays a key role in cell differentiation, proliferation, apoptosis and so on (Ingham and McMahon 2001; Ruiz i Altaba et al. 2007). Previous studies have mainly focused on their specific roles in embryonic development and neural tissue homeostasis (Daya-Grosjean and Couve-Privat 2005). Gli-1 mainly acts as a strong positive activator that enhances the regulation of downstream genes, which is considered as a typical activation marker of the Hh signaling pathway (Gialmanidis et al. 2009). Upregulation of Gli1 significantly enhances the expression of c-myc and vascular endothelial growth factor (VEGF), thereby leading to abnormal cell proliferation and tumorigenesis (Walsh 2005). The Hh signaling pathway is now considered as an important therapeutic target for anti-cancer treatment (Abdel-Rahman 2015; Gao et al. 2015).

MicroRNAs (miRs) are small, non-coding RNAs that repress mRNA translation and induce mRNA degradation through incomplete pairing mechanism by binding to the 3'-untranslated regions (3'UTR) (Zhang et al. 2015). They are widely reported to be dysregulated to human cancers (Gu et al. 2015; Li et al. 2015; Zhang et al. 2015). For instance, miR-128 seems to be involved in multidrug resistance of gastric cancer (Zhang et al. 2015). In addition, miR-449a was reported to inhibit gastric cancer invasion by repressing Flot2 (Li et al. 2015), while abnormal miR-137 expression correlated with gastric cancer progression after radical gastrectomy (Gu et al. 2015).

In this study, we mainly focus on miR-873-5p, which has rarely been studied in previous research. Here, we first reported that Gli1 was a target gene of miR-873-5p. We show novel evidence that miR-873-5p activates the Hh signaling thereby prompting gastric cancer progression.

2. Investigations and results

2.1. *Enhanced Gli1 protein level and decreased miR-873-5p level in gastric cancer tissues*

We first explored the expression of Gli1 in gastric cancer tissues. As shown in Fig. 1A and 1B, the protein level of Gli1 was significantly enhanced in the gastric cancer tissues compared with adjacent noncancerous gastric cancer tissues. In addition, we also found that the level of miR-873-5p was significantly deceased (Fig. 1C).

2.2. *Overexpression of miR-873-5P decreased cell viability, increased cell apoptosis and cell cycle arrest*

To explore the role of miR-873-5p on gastric cancer cell progression, miR-873-5p mimics or inhibitors was transfected into SGC-7901 cells for 24, 48 and 72 h, respectively. As shown in Fig. 2A, overexpression of miR-873-5p significantly decreased cell viability in a time-dependent manner. In comparison, inhibition of miR-873-5p obviously increased cell viability (Fig. 2B). Overexpression of miR-873-5p also induced SGC-7901 cell apoptosis in comparison with negative control (Fig. 2C). In addition, we also found that upregulation of miR-873-5p reduced cell proliferation at G2/M phase in SGC-7901 cells (Fig. 2D).

2.3. *Knockdown of Gli1 induced SGC-7901 cell apoptosis and cell cycle arrest*

A specific siRNA targeting Gli1 was selected. Knockdown of Gli1 significantly enhanced SGC-7901 cell apoptosis as shown by flow cytometry analysis (Fig. 3A). As a result, the proportion of cells

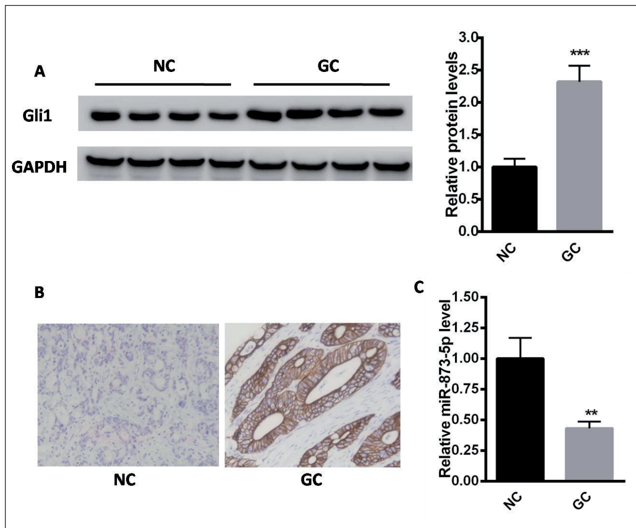

Fig. 1: Decreased miR-873-5P expression and enhanced Gli1 protein level in gastric cancer tissues. The protein level of Gli1 was significantly enhanced in the gastric cancer tissues (GC) compared with adjacent noncancerous gastric cancer tissues (NC) as demonstrated by western blot analysis (A) and immunohistochemistry (B). C. The level of miR-873-5p was significantly deceased. Data were presented as mean ± SE from 3 independent experiments. *P<0.05, **P<0.01.

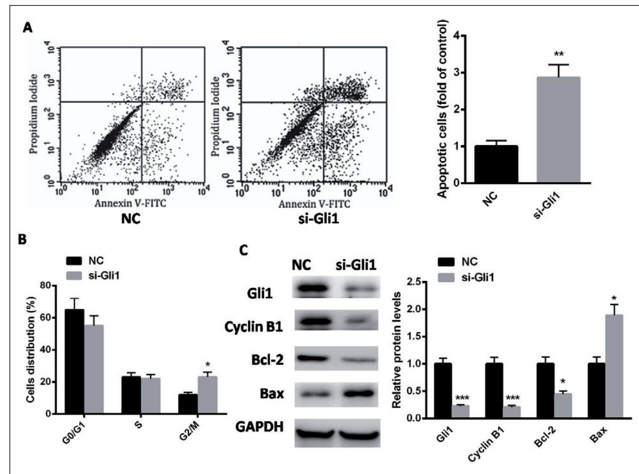

Fig. 3: Knockdown of Gli1 induced SGC-7901 cell apoptosis and cell cycle arrest. A. Knockdown of Gli1 significantly enhanced SGC-7901 cell apoptosis as shown by flow cytometry analysis. B. The proportion of cells at the G2/M stage was increased by 16.75±0.786% in SGC-7901 cells compared with untreated controls. C. Western blot analysis showed that knockdown of Gli1 significantly decreased Cyclin B1, Bcl-2 expression and increased Bax protein level. Data were presented as mean ± SE from 3 independent experiments. *P<0.05, **P<0.01.

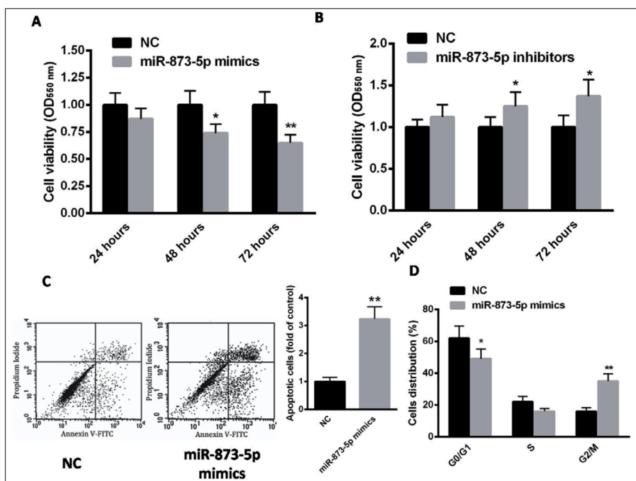

Fig. 2: Overexpression of miR-873-5P decreased SGC-7901 cell viability, increased cell apoptosis and increased cell cycle. A. Overexpression of miR-873-5p significantly decreased cell viability in a time-dependent manner. B. Inhibition of miR-873-5p obviously increased cell viability. C. Overexpression of miR-873-5p also induced SGC-7901 cell apoptosis in comparison with negative control. D. Upregulation of miR-873-5p reduced cell proliferation at G2/M phase in SGC-7901 cells. Data were presented as mean ± SE from 3 independent experiments. *P<0.05, **P<0.01.

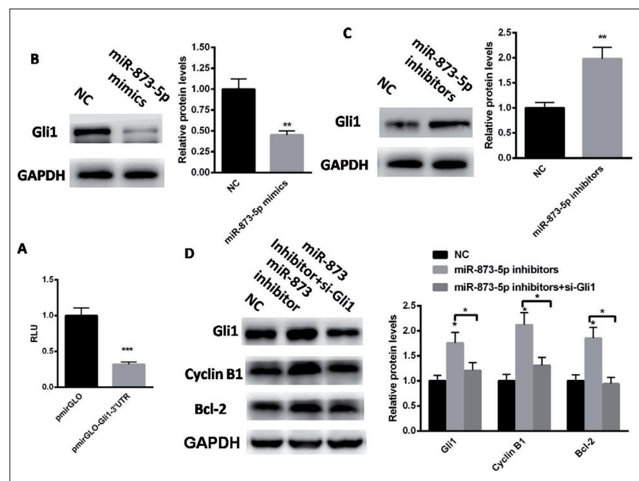

Fig. 4: Gli1 was the target gene of miR-873-5p. A. Overexpression of miR-873-5p significantly decreased the luciferase activity of pmirGLO-Gli1-3'UTR compared with blank vector. Overexpression of miR-873-5p significantly decreased the protein level of Gli1 (B), while inhibition of miR-873-5p obviously increased the expression of Gli1 (C). D. Inhibition of miR-873-5p obviously increased the protein expression of CyclinB1 and Bcl2 even in cells transfected with si-Gli1. Data were presented as mean ± SE from 3 independent experiments. *P<0.05, **P<0.01.

at the G2/M stage was increased by 16.75±0.786 % in SGC-7901 cells compared with untreated controls (Fig. 3B). Further study revealed that knockdown of Gli1 significantly decreased cyclin B1 expression. Meanwhile, the protein levels of Bcl-2 were reduced, while the expression of Bax was increased. These data supported the critical role of Gli1 in the G2/M mitotic/transition phase.

2.4. *Gli1 was the target gene of miR-873-5p*

The 3'UTR of Gli1 containing the binding sites of miR-873-5p was cloned into the pmirGLO plasmid. As shown in Fig. 4A, overexpression of miR-873-5p significantly decreased the luciferase activity of pmirGLO-Gli1-3'UTR compared with blank vector (Fig. 4A). Overexpression of miR-873-5p significantly decreased the protein level of Gli1, while inhibition of miR-873-5p obviously increased the expression of Gli1 (Fig. 4B and 4C). These data suggested that Gli1 was the target gene of miR-873-5p. More importantly, we found that inhibition of miR-873-5p obviously increased the protein expression of CyclinB1 and Bcl2 even in cells transfected with si-Gli1 (Fig. 4D), indicating that inhibition of miR-873-5p enhanced gastric cancer proliferation mainly by targeting Gli1.

3. Discussion

At present, gastric cancer is still one the most common human malignancies (Steeg 2006). Although great advances have been made in diagnosis and therapy, prognosis of gastric cancer is still poor (Hartgrink et al. 2009). It is reported that the 5-year survival rate for gastric cancer patients is only about 20% (de Steur et al. 2015; Haverkamp et al. 2015). The high mortality of gastric cancer patients may result from the metastatic spread of cancer cells into liver and peritoneum (Hartgrink et al. 2000). Thus, it is important to explore the specific mechanism in which gastric cancer is induced. In this study, we found abnormal Gli1 expression in gastric cancer

tissues. Further studies have indicated that Gli1 was a target gene of miR-873-5p. In gastric cancers, decreased level of miR-873-5p led to enhanced expression of Gli1.

In previous studies, excessive Hh signaling was found to be closely related to gastric cancer cell proliferation, migration and invasion (Abdel-Rahman 2015; Samadani and Akhavan-Niaki 2015). In 90 gastric cancer patients, 70% of the gastric cancer tissues demonstrated upregulation of Gli1 expression (Ma et al. 2005). A number of studies have shown that abnormal Hh signaling significantly enhanced cell proliferation and migration in gastric cancer cell lines (Gu et al. 2015). In this study, we confirmed that knockdown of Gli1 decreased the SGC-7901 cell proliferation and enhanced SGC-7901 cell apoptosis. Meanwhile, we found that inhibition of Gli1 induced G2/M cell cycle arrest by repressing cyclin B expression. Moreover, we identified that knockdown of Gli1 induced cancer cell apoptosis. These data indicated the oncogenic role of Gli1 in gastric cancer progression.

Although Hh signaling plays a key role in gastric tumorigenesis, the underlying mechanism is largely unknown. MicroRNAs are small non coding RNAs that widely regulate gene expression. For instance, miR-212 enhanced pancreatic cancer cell proliferation and invasion mainly by targeting patched-1 (Ma et al. 2014). In addition, miR-506 mainly functions as a tumor suppressor which is involved in human cervical cancer progression mainly by targeting Gli3 (Wen et al. 2015). In this study, we first explored the expression of miR-873-5p in gastric cancer cells and found that it was significantly decreased in gastric cancers. Further study found that overexpression of miR-873-5p obviously enhanced gastric cancer cell apoptosis and G2/M cell cycle arrest. Dual luciferase reporter assay indicated that Gli1 was a target gene of miR-873-5p.

In conclusion, decreased miR-873-5p contributed to the abnormal upregulation of Gli1 in gastric cancer tissues. This will not only shed light on the understanding of the molecular mechanism, but also provide a novel potential therapeutic target.

4. Experimental

4.1. Ethics statement

This research was approved by the Ethics Committee of Southeast University, and written informed consent was obtained from each patient involved in the study.

4.2. Human tissue samples

A total of 80 paired cancerous and matched adjacent noncancerous gastric cancer tissues were obtained from gastric cancer patients undergoing gastrectomy at Southeast University from 2011 to 2015. Then, the fresh tissues were treated with RNAlater (Ambion, Inc., USA) which prevents RNA degradation and further frozen at liquid nitrogen. None of these patients had received radiotherapy or chemotherapy prior to surgery. The histopathological type and stage of the gastric cancer were determined according to the criteria of the World Health Organization classification and the TNM stage set out by the Union for International Cancer Control.

4.3. Cell culture

The human gastric cancer SGC-7901 cell line was obtained from American Type Culture Collection (ATCC). The SGC-7901 cells were cultured in RPMI 1640 medium containing 2 mM L-glutamine supplemented with 10% fetal bovine serum (Gibco BRL, Life Technologies Inc., Grand Island, NY, USA), penicillin (100 IU/ml) and streptomycin (100 μg/ml) at 37 ˚C in a humidified atmosphere with 5% CO_2, and harvested with trypsin-EDTA when the cells had reached exponential growth.

4.4. Extraction of total RNA and real-time quantitative RT-PCR

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocol. Then, the total RNA concentration was determined using a NANO DROP spectrophotometer (ND-1000, Thermo Scientific, USA) at OD_{260} nm. Total RNA (1 μg) was transcribed with M-MLV reverse transcriptase (Promega, USA) according to the manufacturer's suggestions. The cDNA was subjected to real-time quantitative RT-PCR for further study. GAPDH and U6 were used as the internal control for mRNA and miRNA expression, respectively. The specific Q-PCR process was listed as follows: 95 °C for 15 s, 60 °C for 15 s and 95 °C for 15 s for 45 cycles. The Ct (threshold cycle) value of each sample was calculated from the threshold cycles with the instrument's software (SDS 2.3), and the relative expression of mRNA or miRNA was normalized to the GAPDH or U6 value using $2^{-\Delta\Delta ct}$ values of each sample.

4.5. Western blotting analysis

The gastric cancer samples or gastric cancer cells were lysed in RIPA lysis buffer (Solarbio, Beijing, China). Then, the lysates were harvested by centrifugation (12,000 rpm) at 4 °C for 30 min. Then, the protein samples were separated by electrophoresis in a 12% sodium dodecyl sulfate polyacrylamide gel and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk for 60 min and further washed with PBST for four times (10 min/time). And the membranes were incubated overnight at 4 °C with specific primary antibodies, Gli1, cyclin B1, Bcl-2 and Bax. The membranes were then washed three times with PBST (10 min/time) and incubated with the horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Zhongshan Gold Bridge Biotech., Beijing, China, all at a 1:2000 dilution) at 37 °C for 1 h. The membranes were washed with PBST for three times and the band density was determined by an enhanced chemiluminescence system (Millipore). The band intensity was determined using the Quantity One software (Bio-Rad Laboratories, Inc. Hercules, CA, USA). The protein levels were normalized to GAPDH (CST, at a 1:10000 dilution).

4.6. Immunohistochemistry

The tissue sections were deparaffinized with dimethylbenzene and rehydrated through 100%, 95%, 90%, 80% and 70% ethanol. After three washes in PBS (phosphate-buffered saline), the slides were boiled in antigen retrieval buffer containing 0.01 M sodium citrate-hydrochloric acid (pH=6.0) for 15 min in a microwave oven. Sections were reacted with rabbit polyclonal anti-Gli1 antibodies (1:200 dilution). Then, the sections were incubated with nonimmune rabbit serum (1:200 dilution) in phosphate-buffered saline (PBS), which is used as negative controls. Then, the cells with positive staining were calculated in at least 10 representative fields (×400 magnification) and the mean percentage of positive cells was calculated. Immunostaining was assessed by two independent pathologists blinded to clinical characteristics and outcomes.

4.7. RNAi and cell transfection

The specific siRNA targeting Gli1 was purchased from Shanghai Jima technology (GenePharma, Shanghai, China). The sequence of siRNAs was listed as follows: 5'-GCCACCAAGCUAACCUCAU GUTT-3' and the nonspecific siRNA sequence of negative control was: 5'-UUCUCCGAACGUGUCACGUTT-3'. Before transfection, 10^6 cells/well were seeded in the six-well plates and the specific siRNA was transfected into SGC-7901 cells using Vigofect transfection reagent (Vigofect, Beijing, China) according to the manufacturer's instructions.

4.8. Transient transfection

Firstly, 6×10^5 cells were equally seeded in the 6-well plates with 2ml DMEM culture medium containing serum and antibiotics. At the same time, miR-875-5p mimics, inhibitors, or miR negative control (Genepharma) were mixed with HiperFect transfection reagent (QIAGEN) and incubated at room temperature for 10 min. Then, the complex was respectively transfected into NCTC1469 cells and Hep1-6 cells for 48 h.

4.9. Cell viability assay

Cell viability was determined using an MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tet- razolium (inner salt; Sigma, M2003) assay. In brief, 5,000 cells were seeded in each well of 96-well plates and incubated with MTS for 24 h. The cell viability was determined at 490 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

4.10. Cell cycle and apoptosis analysis by flow cytometry

Cells were fixed in 70% ethanol and were permeabilized in Triton-X 100. Then, the cells were washed with PBS for three times (5 min/time) and further digested with RNAse A. And the cells were stained with propidium iodide. Apoptotic cells were measured with Annexin V/FITC kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions and analyzed by flow cytometry.

4.11. Statistical analysis

All results are expressed as mean values ± S.E.M. (standard error of mean). The evaluation of real-time PCR data was done by one-way ANOVA with a post-hoc Tukey's test. A value of $P<0.05$ was considered significant.

Conflict of interest: None declared.

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