

MicroRNA-99a promotes cell proliferation, migration and invasion in gastric cancer by modulating c-Src

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Abstract

Background: Recent studies have shown that microRNA-99a(miR-99a) plays a key role in the development of various malignancies; however, its relationship with gastric cancer remains unclear. In this study, we investigated the functions and potential mechanisms of miR-99a in gastric cancer.

Methods: Real-time qRT-PCR was used to assess the expression levels of miR-99a in gastric cancer tissue samples and cell lines compared to their matched adjacent normal tissues and a normal gastric mucosa epithelial cell line, respectively. SGC-7901 cells were transfected with miR-99a mimics and negative controls to determine the effects of miR-99a overexpression on cell proliferation, cell cycle transition, migration and invasion of gastric cancer cells in vitro. The role of miR-99a in endogenous c-Src expression in gastric cancer cells was also investigated by qRT-PCR and Western blotting.

Results: Our results showed a significant increase in miR-99a expression in both gastric cancer tissues and cells compared to normal tissues and cells. Overexpression of miR-99a significantly promoted the cell proliferation, migration and invasion of gastric cancer cells compared to normal cells, with a concurrent increase in the S+G2 phases of the cell cycle. Further investigations found that miR-99a overexpression led to significant upregulation of endogenous c-Src.

Conclusion: Taken together, our findings suggest that miR-99a may act as a tumour promoter in the pathogenesis of gastric cancer by indirectly modulating c-Src expression.

Background

Gastric cancer is one of the most commonly diagnosed cancers worldwide [1, 2]. Although epidemiological evidence has shown that the incidence rates for gastric cancer are steadily declining [3], gastric cancer remains the second leading cause of cancer-related death worldwide [4].

MicroRNAs (miRNAs) are a class of short (~22 nucleotides in length), highly conserved, non-coding RNAs that control the expression of target genes [5]. They downregulate target genes by impairing translation or inducing mRNA degradation by binding through partial sequence homology to the 3'-untranslated region (3'-UTR) of mRNAs [6]. An increasing number of studies have shown that miRNAs participate in the aetiology and pathogenesis of various malignant tumours by targeting oncogenes or tumour suppressor genes [7–9]. These include miR-133b, which functions as a tumour promoter by targeting mammalian sterile 20-like kinase 2 (MST2) in cervical cancer [10]; miR-30a, which functions as a tumour suppressor by targeting metadherin in breast cancer [11]; and miR-29c, which functions as a tumour suppressor by targeting mammalian sirtuin 1 (SIRT1) in hepatocellular carcinoma [12]. The roles of miRNAs in cancer are complex, and a single miRNA can have different biological roles depending on the tumour type; for example, miR-663 was reported to be downregulated and act as a tumour suppressor in gastric cancer [13], whereas it was upregulated and functioned as a tumour promoter in nasopharyngeal carcinoma [14]. A large-scale miRnome study on several miRNAs in six solid tumours showed that miR-218-2 was

downregulated in colon, stomach, prostate and pancreatic tumours but not in lung or breast carcinomas [15].

MiR-99a belongs to the microRNA-99 family, which also includes miR-99b and miR-100. It is attracting increasing attention in cancer studies and has been reported to play a role in the pathogenesis of various malignancies, including head and neck squamous cell carcinoma [16], esophageal squamous cell carcinoma and renal cell carcinoma [17–20]. Although a study on an array of human miRNAs found that miR-99a was significantly upregulated in gastric cancer tissues [21, 22], no further research was carried out.

The purpose of this study was to examine the expression of miR-99a and identify its target genes in gastric cancer tissue samples and cell lines in order to determine its role and underlying mechanisms in the pathogenesis of gastric cancer.

Methods

Gastric cancer tissue samples and cell lines

Human tissue samples were obtained from the surgical specimens of 10 patients with gastric cancer at the Ningbo First Hospital, China. Tumour tissues and their matched adjacent normal tissues were histologically confirmed and preserved in a -80°C freezer after retrieval.

The normal gastric mucosa epithelial cell line GES-1 and three gastric cancer cell lines (SGC-7901, BGC-823 and MGC-803) were obtained from the Cell Bank of Shanghai (China). The cells were cultured in RPMI 1640 supplemented with 10% foetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) and incubated at 37°C in a humidified atmosphere with 5% CO_2 .

Cell transfection

SGC-7901 cells were cultured in 12-well plates. After reaching 30%–50% confluence, transfection was carried out using Lipofectamine 2000 according to the manufacturer's instructions. MiR-99a mimics and negative control oligonucleotide (NC) were designed and synthesized by GenePharma (Shanghai, China). The final concentrations were 160 pM/ml. Total RNA was extracted for qRT-PCR 24 h after transfection. Protein extraction for Western blotting was performed 72 h after transfection. Transfection times for the other assays are as indicated.

Cell proliferation assay

SGC-7901 cells were seeded in 96-well plates at a density of 2000 cells/well and incubated overnight at 37°C , 5% CO_2 . The miR-99a mimics and NC were transfected for 72 h, and 10 μl Cell Counting Kit 8 (CCK-8) solution (Dojindo, Kumamoto, Japan) was added per well. The cells were incubated for an

additional 2 h, and the absorbance was measured at 450 nm using a plate reader (BD Biosciences, San Jose, CA, USA).

Cell cycle assay

The cells were harvested 72 h after transfection and fixed in 70% ethanol at 4°C overnight. The cells were then treated with RNase and stained with propidium iodide (PI) according to the manufacturer's instructions. Cell cycle analysis was performed using a FACSCalibur flow cytometer (BD Biosciences).

Cell migration and invasion assays

SGC-7901 cells were transfected with miRNA-99a mimics and NC for 48 h and then transferred to the upper chamber of a 24-well Transwell system with 8 µm pores. Cells were plated at a density of 1×10^4 cells per well in serum-free Opti-MEM (Gibco). Medium containing 10% FBS was added to the lower chamber as a chemoattractant. A non-coated membrane was used for the migration assay; a membrane pre-coated with Matrigel (BD Biosciences) was used for the invasion assay. After 12–24 h incubation, the cells on the upper surface of the membrane were removed with cotton swabs. Cells that had migrated or invaded to the lower surface were fixed in methanol and stained with 0.2% crystal violet. Micrographs were taken from six random fields for each well.

Real-time quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from the surgical tissue samples and cell lines with TRIzol according to the manufacturer's instructions. The RNA for miR-99a and U6 was reverse transcribed to cDNA with specific RT primers using the M-MuLV First Strand cDNA Synthesis Kit (Sangon Biotech); other RNAs were reverse transcribed to cDNAs using PrimeScript RT Master Mix (Takara, Kyoto, Japan). All primers were designed and synthesized by Sangon Biotech, and the primer sequences are given in Table 1. Real-time quantitative reverse transcription PCR (qRT-PCR) was performed with SYBR Premix Ex Taq II (Takara) using an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA).

Western blot assay

Total protein was collected from the tissue samples and cells using a Total Protein Extraction Kit (Sangon Biotech). Equal amounts of protein per sample were separated by 12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in 5% non-fat milk for 1 h at 37°C and then incubated at 4°C overnight with primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies at 37°C for 1 h. Primary antibodies against c-Src, phospho-c-Src (Tyr416), mTOR, IGF-1R (Cell Signalling Technology, Danvers, MA, USA) and FGFR3 were selected for this study. GAPDH (Cell Signalling Technology) was used as a control. The secondary

antibodies were purchased from Santa Cruz Biotechnology. The immunoreactive bands were detected with ECL reagents and exposed to X-ray film for visualization. The density of each protein band was quantified by normalization to GAPDH.

Statistical analysis

Data from the clinical samples are presented as the median and inter-quartile range in the box plots. Other data are given as the mean \pm SD from at least three independent experiments. The expression levels of miR-99a from the gastric cancer tissue samples and matched adjacent normal tissues were compared by a paired t-test. Other statistical analyses were performed using the independent samples t-test to compare two groups of continuous variables. $p < 0.05$ was considered statistically significant. All analyses were performed using GraphPad Prism (5.01) software.

Results

miR-99a was upregulated in gastric cancer tissue samples and cell lines

The expression levels of miR-99a in both the tissue samples and cell lines were determined by qRT-PCR. The results showed that the average expression level of miR-99a was significantly higher in the 10 gastric cancer tissue samples than in their matched adjacent normal tissues (Fig. 1a). In addition, miR-99a was significantly upregulated in all three gastric cancer cell lines compared to the normal gastric mucosa epithelial cell line (Fig. 1b).

miR-99a promoted the proliferation and affected the cell cycle transition of SGC-7901 cells

To determine the functions of miR-99a in gastric cancer cells, we examined cell proliferation and cell cycle distribution in SGC-7901 cells that were transfected with miR-99a mimics or NC. Both the relative expression of miR-99a and the total number of cells were significantly higher in the miR-99a-transfected cells than in the control cells (Fig. 2a and b, respectively). In addition, the proportion of cells in S+G2 phases of the cell cycle was significantly higher in the miR-99a-transfected SGC-7901 cells than in the control cells (Fig. 2c). Taken together, these findings indicated that miR-99a significantly promoted cell proliferation in SGC-7901 gastric cancer cells and that this may have been linked to G1/(S+G2) phase transitions.

Overexpression of miR-99a promoted migration and invasion in SGC-7901 cells

Transwell migration and invasion assays showed that miR-99a-transfected SGC-7901 cells had significantly higher migration and invasion capacities than control cells (Fig. 3a and b), suggesting that miR-99a could significantly promote cell migration and invasion in gastric cancer.

MiR-99a modulated endogenous c-Src expression in SGC-7901 cells

To explore the mechanisms underlying miR-99a-mediated cell proliferation, migration and invasion, we employed target prediction software (PicTar and TargetScan) and examined the relevant literature to identify potential miR-99a targets. The results of our search showed that the mammalian target of rapamycin (mTOR), fibroblast growth factor receptor 3 (FGFR3) and insulin-like growth factor 1 receptor (IGF-1R) were among the predicted miR-99a targets. Furthermore, the proto-oncogene tyrosine-protein kinase c-Src was reported to be modulated by miR-99a. Therefore, we chose to examine these genes and proteins by qRT-PCR and Western blot assays, respectively. The results showed that c-Src expression was significantly upregulated in miR-99a-transfected SGC-7901 cells compared to control cells at both the mRNA (Fig. 4a) and protein levels (Fig. 4b and c). IGF-1R was only significantly upregulated at the mRNA level (Fig. 4a); however, no significant difference in expression was observed at either level in the other genes (Fig. 4).

Discussion

Several gene expression profiling studies have identified miRNAs that are associated with gastric cancer. These include miR-223, miR-21, miR-23b, miR-222, miR-25, miR-23a, miR-221, miR-107, miR-103, miR-125b, miR-92, miR-146a, miR-214 and miR-191, which were reported to be upregulated in gastric cancer tissue, and let-7a, miR-126, miR-210, miR-181b, miR-197 and miR-30aa-5p, which were found to be downregulated in gastric cancer tissue [22]. MiR-99a has also been reported to play a key role in the pathogenesis of several cancers; however, little is known about its expression or function in gastric cancer. Therefore, we analysed miR-99a expression and its effect in both gastric cancer tissue samples and cell lines.

In agreement with a previous study [21, 22], our findings showed that miR-99a was significantly upregulated in both gastric cancer tissue samples and cell lines compared to their matched adjacent normal tissues and the normal gastric epithelial cell line, respectively. Further studies with miR-99a-transfected cells revealed that overexpression of miR-99a promoted proliferation in SGC-7901 cells, which may have been related to the concurrent increase in G1/(S+G2) phase transitions compared to negative control cells. Furthermore, overexpression of miR-99a significantly enhanced the migration and invasion capacities of SGC-7901 cells. A similar observation was reported for the epithelial-mesenchymal transition in normal mouse mammary gland cells [23]. Conversely, miR-99a has been shown to exhibit the opposite effects in other cancer types by acting as a tumour suppressor. For example, miR-99a overexpression was found to suppress cell growth in hepatocellular carcinoma cells by targeting IGF-1R and mTOR [24], and miR-99a transfection controlled the growth of human

glioblastoma cells *in vitro* by targeting FGFR3 [25]. To elucidate the opposing biological functions of miR-99a in different types of tumours, we examined the effect of miR-99a overexpression on these three genes at the mRNA and protein levels in SGC-7901 cells. IGF-1R was highly expressed at the mRNA level but not at the protein level, and mTOR and FGFR3 showed no significant effect at either level. These results suggested that miR-99a may play different roles in different cancers and act as a tumour promoter in gastric cancer.

In addition to identifying these three miR-99a target genes, we discovered that miR-99a might modulate c-Src expression. Therefore, we carried out further studies by qRT-PCR and Western blotting and found that c-Src was significantly upregulated at both the mRNA and protein levels in SGC-7901 cells overexpressing miR-99a. The proto-oncogene c-Src is a non-receptor tyrosine kinase that phosphorylates and interacts with multiple proteins and protein complexes to mediate intracellular signalling [26], including signalling pathways associated with malignant tumours and tumour progression. Aberrant expression of c-Src has been described in many types of cancer, including gastric cancer [27], and activation of c-Src has been reported to contribute to cell proliferation, survival, adhesion and migration [28, 29]. Therefore, we concluded that miR-99a targeted other genes that influence the expression of c-Src to promote the proliferation, migration and invasion of gastric cancer cells. To confirm this hypothesis, further experiments are required.

Conclusions

In conclusion, our investigations have ascertained the specific biological behaviour of miR-99a in gastric cancer, suggesting that miR-99a may function as a tumour promoter by indirectly modulating the expression of c-Src. These findings could contribute to the understanding of the mechanisms that underlie the pathogenesis of gastric cancer.

Abbreviations

miR-99a: microRNA-99a; qRT-PCR: quantitative real-time reverse transcription PCR; 3'-UTR: 3'-untranslational region; MST: mammalian sterile 20-like kinase 2; SIRT1: sirtuin 1; NC: negative control; CCK-8: Cell Counting Kit 8; PI: propidium iodide; PVDF: polyvinylidene fluoride; mTOR: mammalian target of rapamycin; FGFR3: Fibroblast growth factor receptor 3; IGF-1R: insulin-like growth factor 1 receptor

Declarations

Acknowledgements

Not applicable.

Authors' contributions

The authors have all contributed to this manuscript and approve this submission. LX and PFL: Design of the study; YP: Drafting of the manuscript and Statistical analysis; WXC: Statistical analysis; XY and KSS: Participated in the study concept.

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Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study protocol was approved by the Human Research Ethics Committee of Ningbo First Hospital, Ningbo, Zhejiang, China (approval ID: 2019-R036). Written

informed consent was obtained from each participant.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Table

Table 1 Primers used for qRT-PCR.

| Primers | | Sequences (5'-3') |
|---------|---------|---------------------------|
| miR-99a | Forward | GATAACCCGTAGATCCGAT |
| | Reverse | CAGTGCGTGTCGTGGA |
| U6 | Forward | GCTTCGGCAGCACATATACTAAAAT |
| | Reverse | CGCTTCACGAATTTGCGTGCAT |
| FGFR3 | Forward | CCCAAATGGGAGCTGTCTCG |
| | Reverse | CCCGGTCCTTGTCATGCC |
| IGF-1R | Forward | AGGATATTGGGCTTTACAACCTG |
| | Reverse | GAGGTAACAGAGGTCAGCATTTT |
| mTOR | Forward | GCAGATTTGCCAACTATCTTCGG |
| | Reverse | CAGCGGTAAAAGTGTCCCCTG |
| c-Src | Forward | AGGACCCCAACTGGTACAAAG |
| | Reverse | CGTGGAACCAAGGCATGAG |
| GAPDH | Forward | TCAACGACCACTTTGTCAAGCTCA |
| | Reverse | GCTGGTGGTCCAGGGGTCTTACT |

Figures

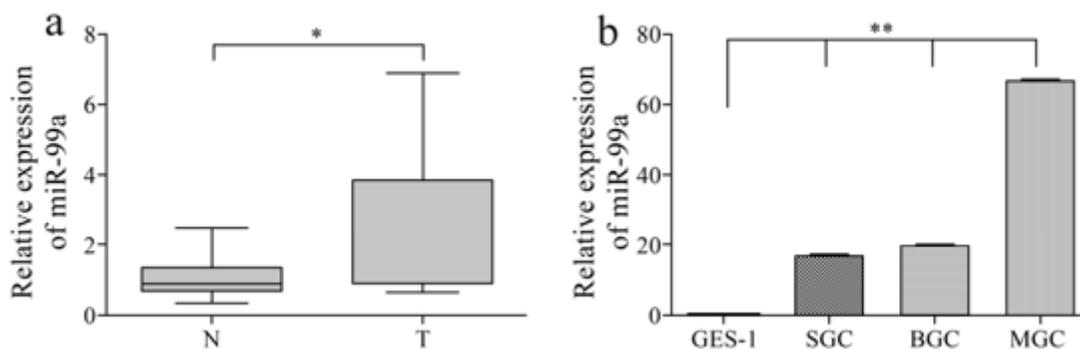


Figure 1

MiR-99a expression in gastric cancer tissues and cell lines. (a) The box plots show that the relative expression of miR-99a is significantly higher in gastric tumour tissues compared to matched adjacent normal tissues. (b) Similarly, miR-99a is significantly upregulated in gastric cancer cell lines (SGC-7901,

BGC-823, MGC-803) compared to the normal gastric cancer cell line (GES-1). T, gastric tumour tissue; N, matched adjacent normal tissue.

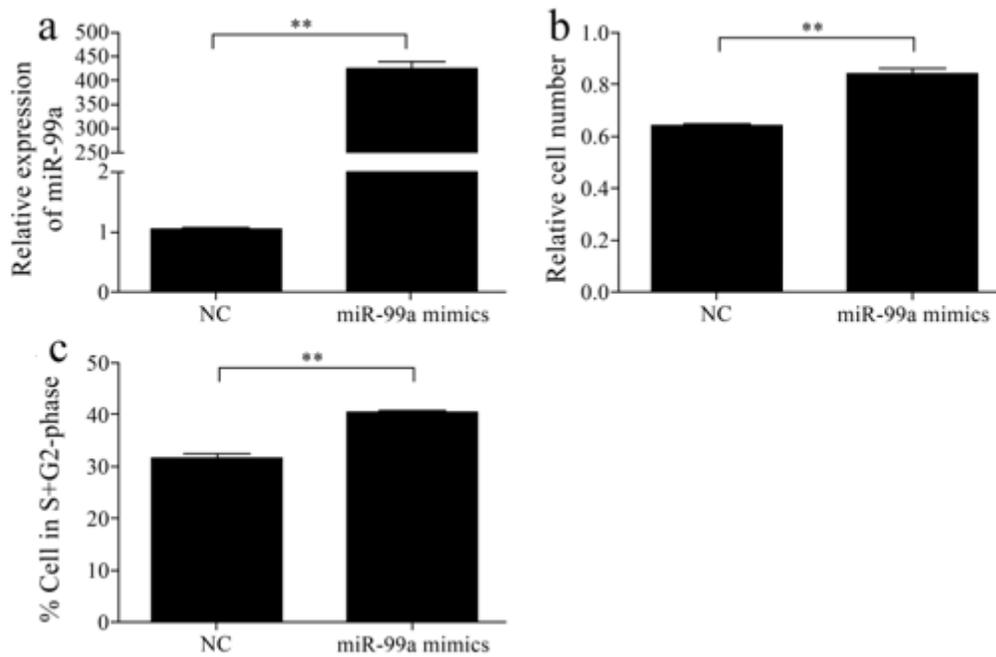


Figure 2

MiR-99a promotes proliferation and alters cell cycle transition in gastric cancer cells. (a) Relative miR-99a expression in SGC-7901 cells transfected with miR-99a mimics was significantly higher than that in negative controls. (b) Overexpression of miR-99a in SGC-7901 cells promotes cell proliferation, (c) potentially leading to G1/(S+G2) phase transitions. NC, negative control.

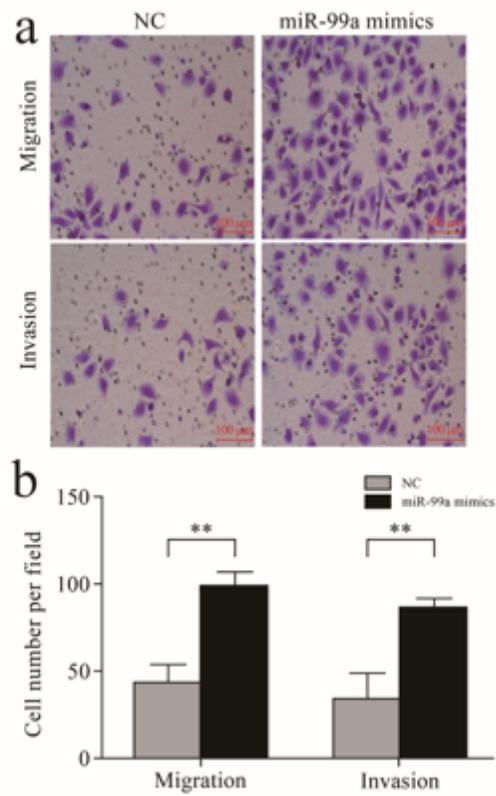


Figure 3

Overexpression of miR-99a promotes migration and invasion in gastric cancer cells. (a) The micrographs show the increased migration and invasion of miR-99a-transfected SGC-7901 cells compared to negative control cells. (b) Quantification based on six random fields for each sample shows that these effects are significant. NC, negative control.

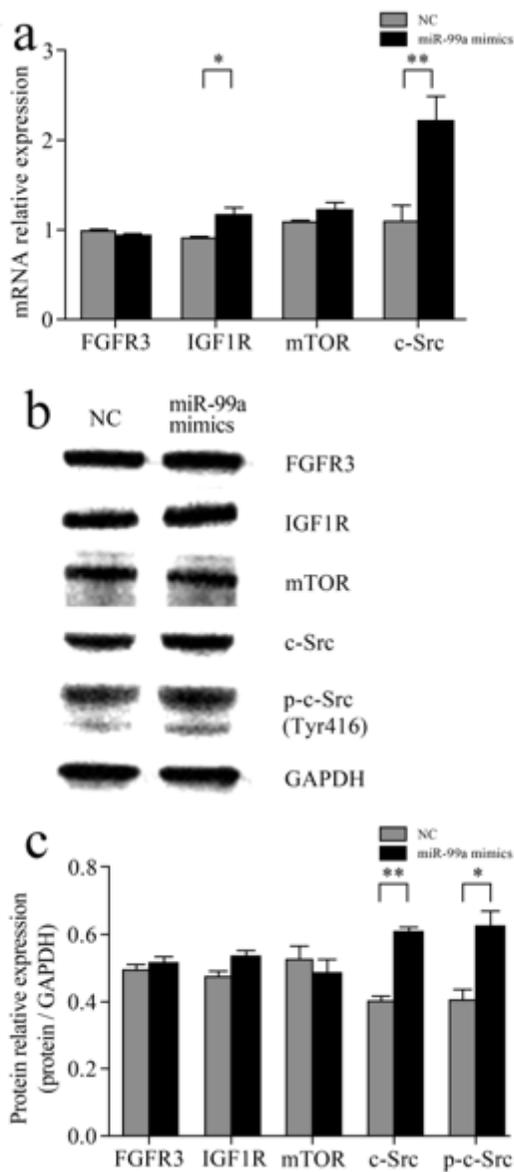


Figure 4

MiR-99a modulates the expression of endogenous c-Src in gastric cancer cells. (a) Both IGF-1R and c-Src are significantly upregulated in miR-99a-transfected SGC-7901 cells at the mRNA level. (b) However, Western blotting shows that only c-Src and phospho-c-Src (Tyr416) are significantly highly expressed at the protein level. NC, negative control.