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Posttranscriptional deregulation of Src due to aberrant miR34a and miR203 contributes to gastric cancer development

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Gastric cancer remains the main cause of cancer death all around the world, and upregulated activation of the nonreceptor tyrosine kinase c-SRC (SRC) is a key player in the development. In this study, we found that expression of Src is also increased in clinical gastric cancer samples, with the protein level increased more significantly than that at the RNA level. Further study revealed that miR34a and miR203, two tumor suppressive miRNAs, inversely correlate with the expression of Src. Restoration of miR34a and miR203 decreased Src expression in gastric cancer cell lines, which in turn inhibited cell growth and cell migration. In summary, our study here revealed that posttranscriptional regulation of Src contributes to the deregulated cell growth and metastasis in gastric cancer, and targeting Src by miR34a or miR203 mimics would be a promising strategy in therapy. [BMB Reports 2013; 46(6): 316-321]

INTRODUCTION

Gastric cancer is one of the most common malignant tumors of the digestive system (1). Due to its genomic instability and the resultant altered gene expression, gastric cancer are of aggressive growth and metastasis ability, and thus remains the leading cause of cancer death all over the world. Elucidating the underlying mechanism, especially the molecular switch for the uncontrolled cell growth and metastasis holds the promise for gastric cancer therapy.

Src-Family Kinases (SFKs) participate in the regulation of proliferation, differentiation, apoptosis, autophagy, adhesion, migration, invasion and angiogenesis in normal and cancer cells. Abnormal expression of SFKs has been documented in cancers that arise in breast, colon, ovary, melanocyte, gastric mucosa, head and neck, pancreas, lung and brain (2). c-Src, the founding member of the SFKs, has been found to be overexpressed or activated in various solid tumors including colon, breast, ovarian, brain cancers and so on. And targeting Src is widely believed to be an optional strategy to cure cancer (3, 4). However, since Src acts both in cancer and normal cells, suggesting that revealing how Src was aberrantly overexpressed and activated is of significant importance for targeting cancer stem cell specifically (5).

Recently, posttranscriptional regulation is believed to play an essential role in development and cancer progress (6). microRNAs (miRNAs) are a class of small non-coding RNAs of 19-25 nt in length, which regulate gene expression at the post-transcriptional level (7, 8). Recent research suggests that miRNAs are involved in tumor invasion and metastasis, in multiple cancers, including gastric cancer (9-11). Among these miRNAs, miR34 and miR203 are characterized as a tumor suppressor in multiple cancers, such as colon cancer and breast cancer by targeting different oncogenes (12-15) However, their roles in gastric cancer is largely unknown.

Here we examined the expression of Src in gastric cancer samples and its relation with miR34a and miR-203. Our study here revealed that Src is increased in clinical gastric cancer samples, while miR34a and miR203, two known miRNAs targeting Src, inversely correlate with the expression of Src. Restoration of miR34a and miR203 decreased Src expression in gastric cancer cell lines, which in turn inhibited cell growth and cell migration. In summary, our study here revealed that posttranscriptional regulation of Src contributes to the deregulated cell growth and metastasis in gastric cancer, and targeting Src using miR34a or miR203 mimics would be a promising

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strategy in therapy.

RESULTS

Increased Src expression both at protein and RNA levels in gastric cancer

Although increased Src activation has been revealed in many cancer types, including gastric cancer (16, 17), its basal expression still needs further confirmation. To this end, we compared the expression of Src in both cancer samples and adjacent normal tissues. As shown in Fig. 1A, Src was found to lowly or moderately expressed in most of the adjacent normal tissues, while its expression in cancer samples was found to be significantly higher. To further clarify how Src expression was increased, we further examined the expression of Src in both cancer and normal tissue samples at RNA level. As expected, Src RNA increased in most cancer samples, compared with their normal counterpart (Fig. 1B), however, the fold change was much smaller, suggesting that posttranscriptional regulation might involve in the process.

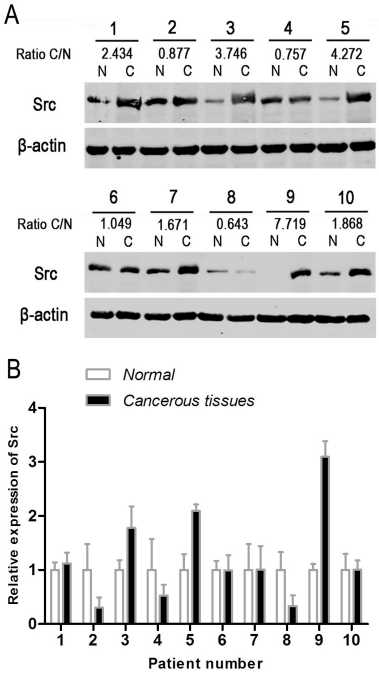

Fig. 1. Increased Src expression in normal and neoplastic gastric epithelium. (A) Protein levels of Src in 10 patients were detected by Western blot and β-actin served as an internal control to ensure equal loading. 10 human gastric cancer specimens and paired adjacent normal tissue collected at surgical resection were processed for protein extraction. (B) RNA levels of Src in the above 10 patients. Expression of Src in both cancer and normal adjacent samples were examined by Real time RT-PCR. β-actin served as an internal control and ΔCt was used for relative expression calculation. The expression of the normal tissue was set as 1. All the experiments were done in triplicate and data were expressed as mean ± SD.

miR34a and miR203 inversely correlates with Src expression

From the above data, we then analyzed the 3′UTR of Src using targetscan (18) and revealed that Src might be targeted by miR34 and miR203 (Fig. 2A). Further literature searching further confirmed that miR203 are the posttranscriptional regulators of Src (19, 20). In this regard, we test the expression of miR34a and miR203 expression in the above cancer samples. Strikingly, both miR34a and miR203 were significantly downregulated in cancer samples, which was inversely correlated with the expression of Src in cancer samples (Fig. 2B and C). We next test whether miR34a and miR203 could really target Src in gastric cancer. The available gastric cancer cell lines were screened for miR34a, miR203 and Src expression. Among these cells, MKN45 cell displayed moderate expression of the above molecules (data not shown). In this regard, we transfected either miR34a or miR203or their antagonisms respectively and tested the expression of both Src and phosphorylated Src. Efficient transfection were confirmed by qRT-PCR (Supplementary Fig. 1A and B). As expected, transfection of miR34a or miR203 repressed Src expression and subsequent its activity (Fig. 2D and E). In contrast, inhibiting endogenous miR34a or miR203 function by transfection of their antagonisms further increased Src expression and activity in the same cell (Fig. 2D and E). Luciferase activity assay revealed that either miR34a or miR203 inhibited the 3'UTR of Src activity, while their antagonisms increased 3'UTR activity of Src (Fig. 2F).

miR34a and miR203 inhibit the cell growth and migration in Src dependent manner

All of the above findings suggest that miR34a and miR203 might be the main cause of deregulated Src, and it might be also responsible for the deregulated Src function in cancer. As expected, knockdown of Src by different RNAi reduced Src expression and its activation in MKN45 cells (Fig. 3A). With the Src expression decreased, migrating cell numbers reduced significantly (Fig. 3B and C). Similar as knockdown of Src, transfection of miR34a decreased the cell growth (Fig. 4A) and reduced the cell migration (Fig. 4B and C). In contrast, inhibiting miR34a had an opposite effect on cell migration and proliferation. While inhibiting miR34a by antago-miR34a nearly has no effects on cell proliferation and migration when cells were together treated with si-Src (Fig. 4A-C), suggesting that Src is the main target of the tumor suppressor role of miR34a. Similar as miR34a, miR203 plays a similar role on cell proliferation and migration (Fig. 4D-F).

DISCUSSION

Gastric cancer remains the main cause of cancer death worldwide, as the conventional strategies based on radical surgery for the treatment of gastric cancer are not yet satisfactory.

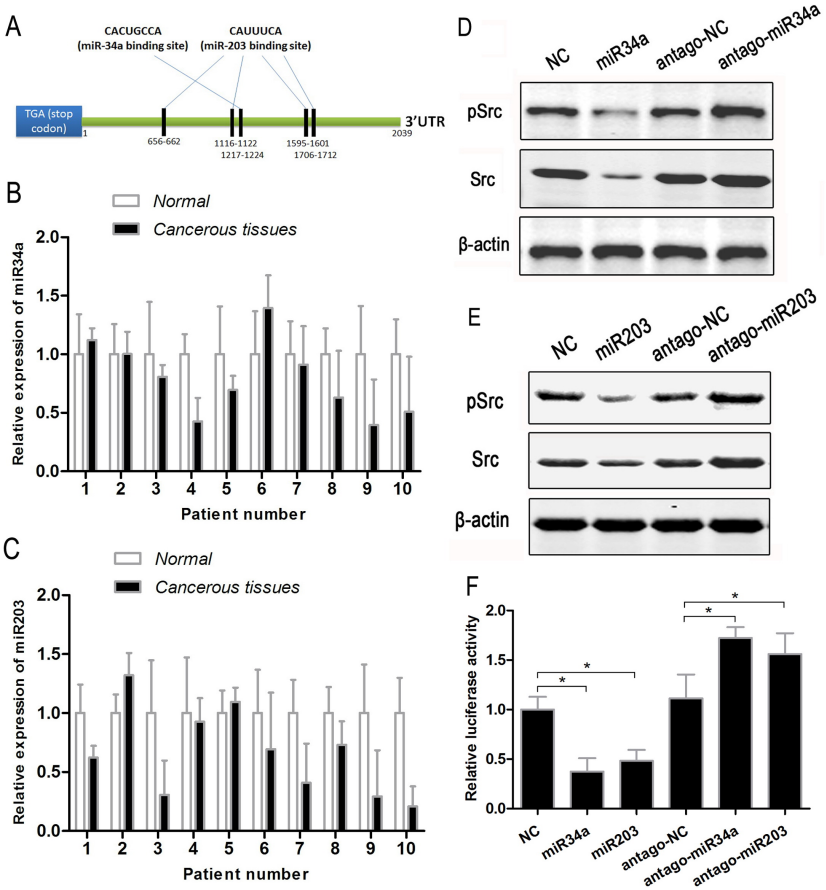

Fig. 2. Expression of miR34a and miR203 inversely correlates the expression of Src. (A) Src mRNA 3′UTR was analyzed by the online tool targetscan and the loci for putative miR34a and miR203 recognition is indicated. (B) miRNA levels of miR34a in the above 10 patients. Relative expression of miR34a in both cancer and normal adjacent samples were examined by Real time RT-PCR. U6 served as an internal control. All the experiments were done in triplicate and data were expressed as mean ± SD. (C) miRNA levels of miR203 in the above 10 patients. Relative expression of miR203a in both cancer and normal adjacent samples were examined by Real time RT-PCR. U6 served as an internal control. All the experiments were done in triplicate and data were expressed as mean ± SD. (D) MKN45 cells were transiently transfected with the control, miR34a or its antagonism. Expression of the endogenous Src and phosphorylated Src was analyzed by Western Blot. Data presented here is a representative of three different experiments. (E) MKN45 cells were transiently transfected with the control, miR203 or its antagonism. Expression of the endogenous Src and phosphorylated Src was analyzed by Western Blot. Data presented here is a representative of three different experiments. (F) MKN45 cells were transfected with 3'UTR of Src luciferase reporter and TK-Renilla reporter as an internal control, indicated miRNA and the antagonism was also co-transfected. Twenty-four hours later, relative luciferase was determined after normalization for Renilla (*$P < 0.05$).

Therefore, investigation of the mechanisms of uncontrolled cell growth and metastasis are attracting increased attention in both basic and clinical cancer research. In this study, we for the first time revealed that miR34a and miR203 decreased Src expression in gastric cancers, which in turn inhibited cell growth and cell migration, and their deregulated expression leads to increased Src expression and activation.

Src is found to be over activated in many cancers, including gastric cancer, and therapeutic strategies by targeting c-Src hold promise for these kind of cancers (17). Dasatinib inhibits the activity of c-Src and several other kinases. Although it holds promise for cancer therapy, the obvious side-effects suggest the need to reveal the mechanism how c-Src is specifically activated in gastric cancer,which might shed light on new strategies for Src inhibition. Recently, microRNAs, has received significant attention in cancer research and aberrant expression of oncogenic miRNAs is associated with the development and progression of many cancers, including gastric cancer (21). However, the deregulated miRNAs and the mechanisms by which miRNAs affect oncogenesis remain to be elucidated. In the present study, we showed that miR203 and miR34a are down-regulated in gastric cancer compared with the normal counterpart. Moreover, we showed that the over-expression of miR203 and miR34a could suppress the

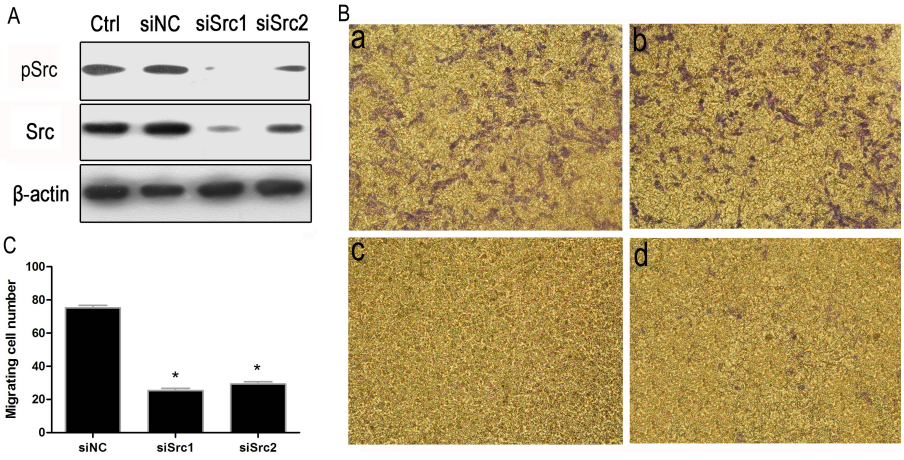

Fig. 3. Knockdown of Src reduced the cell migration. (A) Efficiency of Src knocking down. Two different RNAi duplexes targeting Src or the negative control RNAi were transfected into MKN45 cells cultured in 6-well plate for 48 h. Knockdown were efficiently realized as detected by Western blot. (B) Knockdown of Src inhibits the cell migration. MKN45 cells transfected with indicated treatment (a, no treatment; b, siNC; c, siSrc1; d, siSrc2) were seeded on the transwell for migration analysis 24 after transfection. Data presented here is a representative of different experiments. (C) Statistical data of (B) (*P < 0.05).

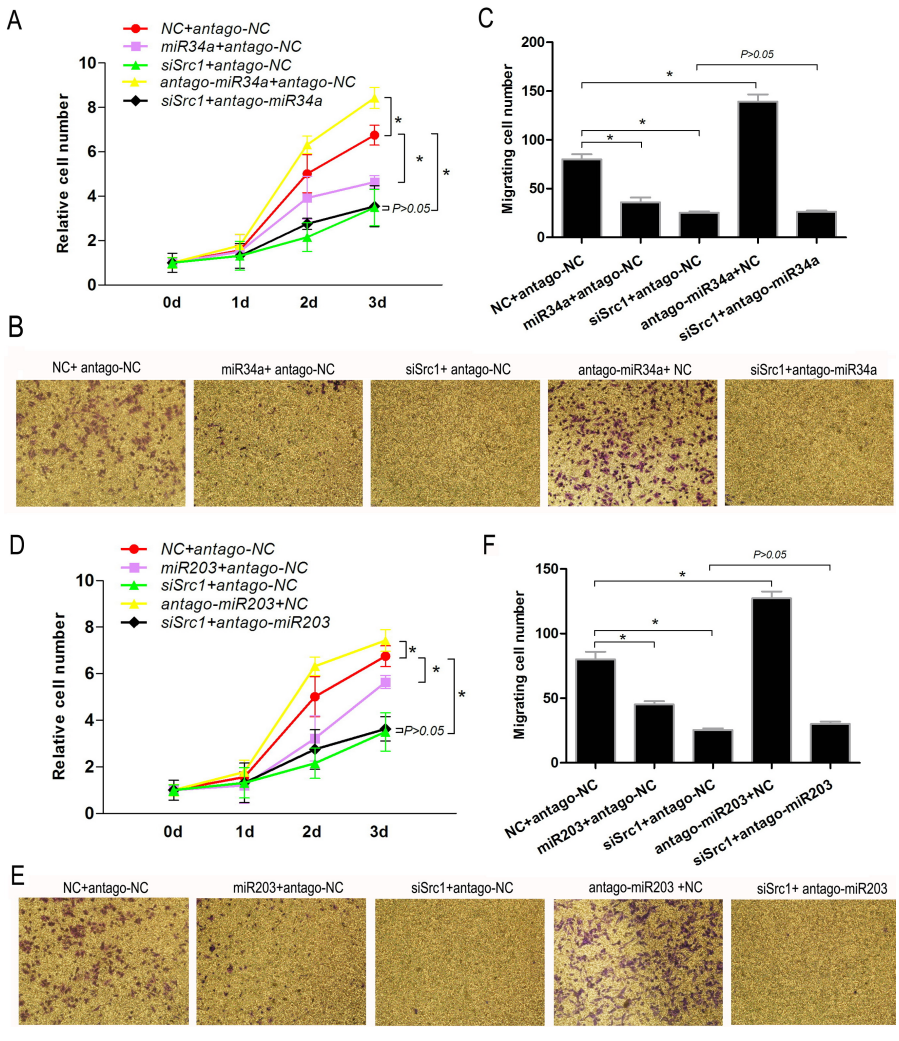

Fig. 4. miR34a and miR203 inhibit cell proliferation and migration in a Src dependent manner. (A) MKN45 cells transfected with either miR34a, Src RNAi, antago-miR34a or their combinations. Twelve hours after transfection, cells were seeded on 96-well plate and harvested for MTT assay at different time. miR34a inhibits cell growth while its antagonism promotes cell growth. Antago-miR34a had no obvious effect in cells with Src knockdown. Experiments were done in triplicate and data were expressed as mean ± SD. (B) Cells treated same as above were seeded on transwell for migration analysis. Data presented here is a representative of different experiments. (C) Statistical data of (B) (*P < 0.05). (D) MKN45 cells transfected with either miR203, Src RNAi, antago-miR203 or their combinations as indicated were seeded on 96-well plate and harvested for MTT assay at different time (*P < 0.05). (E) Cells treated same as above were seeded on transwell for migration analysis. Data presented here is a representative of different experiments. (F) Statistical data of (E) (*P < 0.05).

proliferation and migration of gastric cancer cells, accompanied by a decrease in the expression of Src, suggesting that both miR34a and miR-203 have tumor-suppressive effects in gastric cancer.

Consistently, both miR34a and miR203 are down regulated in several cancer cells. Taken together, these results suggest that miR34a and miR203 may act as a tumor suppressor. In fact, miR34a and miR203 might together contribute to the deregulated Src expression, as either miR203 or miR34a could not absolutely mimic the effects of RNAi against Src. It is important to note that individual miRNAs are capable of regulating dozens of distinct mRNAs, so we considered the possibility that either miR34a or miRNA203 might act on several other target genes besides Src. In fact, recently miR203 was found to target BIRC5 and LASP1 (15), and miR34a could target E2F1 and B-Myb oncogenes in leukemic cells (22). In other words, our study here revealed that Src is one of the most important targets of miR34a and miR203, while we could not exclude other important targets.

Posttranscriptional regulation usually acts as a positive or negative feedback in the circuit of cancer regulation (23). And targeting the components of the circuit is of great therapeutic potential, while miRNAs are of extremely interest as they are so small for *in vitro* mimicking and *in vivo* delivery.

In summary, we here revealed that aberrant downregulation of miR34a and miR203 are at least partially the main reason for increased Src expression and the subsequent activation in gastric cancers, and targeting Src by miR34a or miR203 mimics would be a promising strategy in therapy.

MATERIALS AND METHODS

Clinical sample collection

Ten cases of gastric cancer samples and the adjacent normal specimens were obtained from the 323 hospital of PLA (Xi'an, China). All eligible cases had not received preoperative tumor-related treatment. Normal gastric mucosa tissues were taken from the tumor marginal zone (>5 cm from the tumor). And cancer samples were confirmed by sophisticated pathologist. Ethical approval was obtained from the Fourth Military Medical University Research Ethics Committee.

Cell culture

Human gastric cancer cell lines, MKN45, were purchased from the Cell Bank of the Chinese Academy of Sciences. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum in 5% CO_2 at 37°C.

Western blot

Tissue and cell samples were lysed with RIPA buffer containing proteinase inhibitor, PMSF (1 mM), Na3VO4 (1 mM), NaF (1 mM). Protein concentration was determined by the BCA protein assay (Pierce Chemical Co.). Equal amounts of cell lysates were separated on SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes. Membranes were then incubated in blocking solution (5% non-fat milk in 20 mM TRIS-HCl, 150 mM NaCl, 0.1% Tween-20) (TBS-T), followed by incubation with the indicated antibodies (anti-Src and anti-β-actin from Santa Cruz, Anti-p-Src from Abcam) at 4°C overnight. The membranes were then washed in TBS-T and incubated with HRPO-conjugated secondary antibodies for 1 hour at room temperature. Antibody detection was performed with an enhanced chemiluminescence reaction.

qRT-PCR

For target mRNA detection, the target specific primers and endogenous control GAPDH were synthesized by Sangon (Shanghai, China) (Supplemental Table 1). And for miRNA detection, the sequence-specific primers for miRNAs and endogenous control U6 snRNA were synthesized (Supplemental Table 1). The reverse-transcription reaction was carried out with M-MLV (Promega) or miRNA reverse transcription kit (Qiagen) according to the manufacturer's instructions. Real-time quantification polymerase chain reaction (qPCR) analysis was carried out after cDNA was synthesized. Reaction conditions were as follows: 95°C for 10 minute; followed by 40 cycles of 95°C for 15 second, 65°C for 5 second, and 72°C for 45 second for the amplification. The gene expression Ct values of miRNAs/mRNA from each sample were calculated by normalization to the internal control U6/GAPDH snRNA. All the experiments were done in triplicate.

Transfection

Cells were were seeded at 50% confluence and 16 hours after seeding, cells were transfected with 20 ul Opti-MEM (Invitrogen) and the indicated amount of RNAi/miRNA. Cells were subjected to MTT or migration assay after transfection at indicated time.

Luciferase assays

Src 3'UTR was amplified and cloned into the pGL3-control vector using indicated primers (Supplementary Table 1). To avoid self-assembly, the Xba1 digested pGL3-control vector was further treated with CIP to remove 5´ phosphates before ligation. The clone denoted as pGL3-Src 3'UTR was confirmed by sequencing to insure right direction. pGL3-Src 3'UTR cells and the internal control TK vector were contransfected into MKN45 cells, together with indicated miRNAs or their antagonism similar as described (24). Relative luciferase activity was analyzed as instructed 24 hours after transfection.

MTT assay

MTT test was used to assess cell proliferation, the. Briefly, cells with different treatments were seeded at a density of 1,000 cells/well in 96-well plates. 3-(4,5-methylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) was added (100 μg/well) for 4 hours at the indicated time. Formazan products were solubilized with DMSO, and the optical density was measured

at 490 nm. All experiments were performed in triplicate.

Cell migration assay

Cells were harvested and re-suspended in serum-free medium. For the migration assay, 5×10^4 cells were added into the upper chamber of the insert (BD Bioscience, 8 μm pore size). Cells were plated in medium without serum above the chamber. And in the lower chamber, medium containing 10% fetal bovine serum was served as the chemoattractant. After 6 hour of incubation, cells were fixed with 3.7% formaldehyde and stained with crystal violet staining solution, and cells on the upper side of the insert were removed with a cotton swab. The migratory capacity was evaluated as the total number of cells on the lower surface of the membrane, as determined by microscopy.

Statistical analysis

Statistical analysis was performed using one-way ANOVA or Student's t test. Values of $P < 0.05$ were considered significant. Data were represented as the mean $\pm$ S.D. GraphPad Prism 5.0 software was used for all data analysis.

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