MiR-193a-5p and -3p Play a Distinct Role in Gastric Cancer: miR-193a-3p Suppresses Gastric Cancer Cell Growth by Targeting ETS1 and CCND1

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Abstract. *Background/Aim:* MicroRNAs (miRNAs) are small non-protein-coding RNAs, that can be generated from the 5p or 3p arm of precursor miRNA (pre-miRNA). Differential miRNA arm selection has been reported between tumor and normal tissue in many cancer types; however, the biological function and mechanism of miRNA arm switching in gastric cancer remain unclear. *Materials and Methods:* Profiles of miRNA expression in gastric cancer were obtained from The Cancer Genome Atlas (TCGA). The biological role of miR-193a-5p/-3p in tumor growth and invasive abilities was assessed through a gain-of-function approach. Target genes of miR-193a-3p were identified using bioinformatics and an experimental approach. *Results:* The expression levels of miR-193a-5p, and not of miR-193a-3p, were significantly decreased in gastric cancer compared to adjacent normal tissues. Ectopic expressions of miR-193a-5p and miR-193a-3p revealed that they both inhibited gastric cancer cell growth, but only miR-193a-3p significantly suppressed cell invasion ability. Using a bioinformatics approach, we identified 18 putative target genes of miR-193a-3p. Both mRNA and protein levels of cyclin D1 (CCND1) and ETS proto-oncogene 1

(ETS1) were significantly decreased in AGS cells transfected with miR-193a-3p mimics. ETS1 or CCND1 knockdown significantly suppressed gastric cancer cell growth, similar to miR-193a-3p overexpression. *Conclusion:* Our results indicated that miR-193a-3p suppressed gastric growth and motility, at least partly, by directly targeting CCND1 and ETS1 expression.

Gastric cancer is the fourth most common cancer and the second leading cause of cancer death, worldwide (1). Some of the known risk factors demonstrated to accelerate gastric cancer progression include *Helicobacter pylori* infection, dietary factors, tobacco use, and alcohol consumption (1). The prognosis and survival of gastric cancer patients strongly depend on stage and metastasis status. However, because of the lack of highly sensitive and specific biomarkers, gastric cancer tends to be identified at advanced stages in most patients. Gastric carcinogenesis is a complex multistep process in which DNA mutation, gene expression, and DNA epigenetic modification are quantitatively altered during cancer progression (2-5). These gene and genetic dysfunctions could be candidates as diagnostic or prognostic markers for gastric cancer (5, 6).

MicroRNAs (miRNAs) are small non-protein-coding RNAs; they play a crucial role in the progression of several diseases, including cancer (7). Our previous studies have reported that miRNAs-miR-9, -34b, -129, and -196a/b –are suitable as biomarkers for the diagnosis or progression of gastric cancer (8-11). Mature miRNA is generated from a precursor miRNAs (pre-miRNAs), which are approximately 70 nucleotides in length and comprise a 5p arm, a 3p arm,

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Key Words: miR-193a, ETS1, CCND1, gastric cancer, arm selection.

and a terminal loop. An identical pre-miRNA can generate either miR-#-5p or miR-#-3p through Dicer processing (12-14). The preference of 5p or 3p arm depends on the hydrogen bond theory (15). Recent studies have shown that target-mediated miRNA protection (TMMP) also contributes to miRNA arm switching (16, 17), leading to changes in the miRNA arm selection during the progression of cancers including gastric cancer, breast cancer, and hepatocellular carcinoma (18-20). However, the biological function and mechanism of miRNA arm switching in gastric cancer remain unclear. In this study, we identified miRNA candidates with arm switches by analyzing The Cancer Genome Atlas (TCGA) database. Furthermore, miR-193a-5p and -3p were selected for the assessment of their biological function in gastric cancer.

Materials and Methods

Expression data from the TCGA database. All level-3 expression data of breast cancer were downloaded from the TCGA database portal (<https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm>). The miRNA level-3 data of 399 gastric cancer tissues and 45 corresponding adjacent normal tissues were obtained. In addition, the level-3 data of the RNA transcriptome profile from 415 gastric cancer tissues and 35 corresponding adjacent normal tissues were obtained.

Cell lines. Human gastric cancer cell lines, AGS, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted using a TRIzol reagent (Invitrogen), according to the instruction manual. Samples were briefly homogenized in 1 ml of TRIzol reagent and mixed with 0.2 ml of chloroform to extract protein. RNA was precipitated using 0.5 ml of isopropanol. The concentration, purity, and amount of total RNA were determined using a Nanodrop 1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE, USA).

Ectopic expression of miR-193a-5p and miR-193a-3p. AGS were transfected with 10 nM of miRNA -193a-5p mimics, miR-193a-3p mimics, or the appropriate miRNA mimics control (GenDiscovery Biotechnology, Inc, Taipei, Taiwan) using Lipofectamine RNAiMAX Reagent (Invitrogen). After 24 h of transfection, the transfected cells were harvested, and the expression levels were examined by stem-loop for reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR).

Stem-loop RT-qPCR. A total of 1 µg of total RNA was reverse-transcribed in a stem-loop reverse transcription (RT) reaction by using RT primers and SuperScript III Reverse Transcriptase according to the user's manual (Invitrogen). The reaction was performed under the following incubation conditions: 30 min at 16°C, followed by 50 cycles of 20°C for 30 s, 42°C for 30 s, and 50°C for 1 s. The enzyme was subsequently inactivated through incubation at 85°C for 5 min. Real-time polymerase chain reaction (qPCR) was performed using a miR-193a-5p and miR-193a-3p-specific forward primer and a universal reverse primer. The reaction was conducted at 94°C for 10 min, followed by 40 cycles of 94°C

for 15 s and 60°C for 32 s. The gene expression level was detected using the SYBR Green I assay (Applied Biosystems, Foster City, CA, USA), and the expression levels of miR-193a-5p or miR-193a-3p were normalized to the expression of U6 non-coding small nuclear RNA. The primer sequences used to examine miRNAs are shown in Table I.

qRT-PCR. In total, 2 µg of total RNA were reverse-transcribed with oligo (dT)15 primers and SuperScript III Reverse Transcriptase according to the user's manual (Invitrogen). The reaction was performed with incubation at 42°C for 1 h; then, the enzyme was subsequently inactivated by incubation at 85°C for 5 min. The cDNA was used for the qPCR analysis with gene-specific primers, and gene expression was detected using the SYBR Green I assay (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used for internal control. The sequences of ETS proto-oncogene 1 (ETS1), Cyclin D1 (CCND1), and GAPDH are shown in Table I.

Western blotting. The cells were harvested 24 h after transient transfection; cells were washed with phosphate-buffered saline (PBS) and then lysed with lysis buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1% NP-40, 0.02% sodium azide, 1 µg/mL aprotinin, 1 mM PMSF) at 4°C for 30 min. The lysate was collected and centrifuged to remove cell debris. Protein assays were performed using the Bio-Rad Protein Assay kit based on the Bradford dye-binding procedure (Bio-Rad, Hercules, CA, USA). Protein samples (60 µg) were separated by SDS-PAGE in 10% resolving gel using a Mini-PROTEAN 3 Cell apparatus (Bio-Rad). Proteins were then electrotransferred to polyvinylidene difluoride (PVDF) membranes (NEF1002001PK, PerkinElmer, Inc., Waltham, MA, USA). After blocking at 4°C overnight using PBS-Tween containing 5% skim milk, membranes were incubated with anti-CCND1 (1:200, RM9104S, Thermo Fisher Scientific Inc., Waltham, MA, USA), anti-ETS1 (1:100, sc-55581, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-beta actin (ACTB; 1:2000, MAB1501, EMD Millipore, Billerica, MA, USA) for 1 h in PBS-Tween containing 5% skim milk. Membranes were then incubated with anti-rabbit (sc-2004) or anti-mouse (sc-2005) IgG HRP-conjugated secondary antibodies (1:10000, Santa Cruz Biotechnology) for 1 h at room temperature. After three washes with PBS-Tween, immunoreactive bands were detected using ECL kit (Advansta, Menlo Park, CA, USA).

ETS1 and CCND1 knockdown with siRNA. Small interfering RNA (siRNA) oligonucleotides targeting ETS1, CCND1 and a scrambled oligo as a negative control were designed and synthesized by GenDiscovery Biotechnology (Taipei, Taiwan). The detailed information is shown in Table I. AGS cells were transfected with a final concentration (10 mM) of individual siRNA or control using Lipofectamine RNAiMAX (Invitrogen; Thermo Fisher Scientific). After transfection for 24 h, the protein was extracted, and knockdown efficiency was evaluated through western blotting.

Cell proliferation and invasion assay. For cell proliferation analysis, 2000 AGS cells were plated onto the 96-well plates. Cell growth was determined at 0, 1, 2, 3, and 4 days using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA). Cells were tested for invasion abilities *in vitro* in a Transwell chamber (Costar, Lowell, MA, USA). The lower side or the upper side of the polycarbonate

Table I. Sequences of the primer sets and small interfering RNAs (siRNAs).

Primer	Primer sequences
miR-193a-5p-RT	5'-CTCAACTGGTGTCTGGAGTCGGCAATT CAGTTGAGTCATCTCG-3'
miR-193a-5p-GSF	5'-CGGCGGTGGGTCTTTGCGGGCG-3'
miR-193a-3p-RT	5'-CTCAACTGGTGTCTGGAGTCGGCAATT CAGTTGAGACTGGGAC -3'
miR-193a-3p-GSF	5'-CGGCGGAAGTGGCCTACAAAGT-3'
Universal reverse	5'-CTGGTGTCTGGAGTCGGCAATTC-3'
U6-F	5'-CTCGCTTCGGCAGCACA-3'
U6-R	5'-AACGCTTCACGAATTTGCGT-3'
ETS1-F	5'-CTCAGATATGGAATGTGCAG-3'
ETS1-R	5'-TGCTGTTCTTTAGTGAAC-3'
CCND1-F	5'-CGCCCCACCCCTCCAG-3'
CCND1-R	5'-CCGCCCAGACCCTCAGACT-3'
GAPDH-F	5'-TGCACCACCAACTGCTTAGC-3'
GAPDH-R	5'-GGCATGGACTGTGGTCATGAG-3'
siRNA	siRNA sequences
si-ETS1	sense: 5'-GCAUUAAGCUUUUUCATT-3' antisense: 5'-UGAAAGUAGUUUUAUGCTT-3'
si-CCND1	sense: 5'-GGAGCAUUUUGAUACCAGATT-3' antisense: 5'-UCUGUAUCAAUCCGCG-3'

RT, GSF, F, R

membranes (8- μ m pore) of the Transwell chambers were coated with 0.5 μ g/ μ l of Matrigel and were used for invasion assays. Cells were added to the upper chamber of the Transwell chamber. After incubation for 18 h at 37°C, the cells at the lower side were prepared for Giemsa staining. The level of invasion was determined using a microscope at 200 $\times$ magnification. All experiments were repeated three times.

Soft agar assay. The base agar layer was prepared with 0.5% agar (Laboratorios CONDA, Torrejón de Ardoz, Madrid, Spain) dissolved in 1.5 ml of culture medium on 6-well plates; subsequently, 1.5 ml of 0.35% agarose (Invitrogen, Grand Island, NY, USA) solution containing the cells (20,000 cells/well) with CCND1 or ETS1 knockdown was inoculated on top of the base agar layer. After allowing the solution to harden, 2 ml of fresh medium were added to the top of the hard agar layer. After 2-3 weeks, agar plates were stained with iodinitrotetrazolium chloride (SC-203739, Santa Cruz Biotechnology) and the colonies were counted.

Colony formation assay. A total of 4,000 cells were seeded on each well of a 6-well plate, and the cells were transfected with individual miR-193a-5p mimics, miR-193a-3p mimics, si-CCND1, si-ETS1, or a control using Lipofectamine RNAiMAX (Invitrogen; Thermo Fisher Scientific). After incubation at 37°C for 3 days, the cultured medium was replaced with a new medium. The cells were incubated at 37°C for 14 days. Cell culture plates containing colonies were fixed with 4% formaldehyde for 2 min, and the colonies were stained with crystal violet solution for 2 h. The wells were rinsed with water, and after air-drying, crystal violet stain was solubilized

by adding 1 ml of 10% acetic acid per well. The absorbance (optical density) of the solution was measured on a spectrophotometer at a wavelength of 595 nm.

Candidate miRNA targets and assay of luciferase activity. Putative target genes of miR-193a-3p were predicted using the TargetScan tool (release no. 7.0) (21). In this study, we identified 76 candidate genes for miR-193a-3p targeting. The 3'UTR sequences of ETS1 were cloned into the pMIR-REPORTTM vector (AM5795; Thermo Fisher Scientific). Then, the pMIR-REPORT-ETS1 was cotransfected with or without miR-193a-3p mimics into gastric cancer cell line using Lipofectamine 2000 (Invitrogen). After 24 h of transfection, cell lysates were used for measuring luciferase activity using the Dual-Glo Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Statistical analysis. The ratio of miRNA-#-5p/miRNA-#-3p between gastric cancer and adjacent normal tissue from the TCGA database were analyzed using Student's *t*-tests. The correlation of the miRNA-#-5p/-3p ratio between the gastric cancer and normal tissue was determined through Pearson's coefficient analysis, with *r* and *p*-values as indicated. The expression levels of miR-193a-5p or miR-193a-3p in paired gastric tissues were analyzed using a paired *t*-test. The expression levels of CCND1 and ETS1 were examined in gastric cancer and adjacent normal tissues from TCGA database using Student's *t*-tests. Experiments for luciferase reporter assay, cell proliferation assay, colony formation assay, soft agar assay and invasion were conducted in triplicate. Histograms present the mean values, and the error bars indicate the standard deviation (SD). These data were analyzed using Student's *t*-tests. Differences with *p*<0.05 were considered statistically significant.

Results

Identification of miRNA arm switching using the TCGA database. TCGA database was used to analyze miRNA arm switching changes in gastric cancer. First, the small RNA-seq database, which contains 399 gastric cancers and 45 adjacent normal tissues from TCGA database, was downloaded (Figure 1A). After analyzing these miRNA profiles, 261 pre-miRNAs could generate both miRNA-#-5p and miRNA-#-3p in gastric cancer. As shown in Figure 1B, arm selection preference in most of the miRNAs was consistent ($R^2=0.991$) between gastric cancer and adjacent normal tissue (Figure 1B). Only a few cases were observed in which the selection of the 5p and 3p arm had different preferences between gastric cancer and adjacent normal tissue (fold change>1.5 or <0.75; *p*<0.01) (Figure 1A). The miRNA-#5p/miRNA-#3p ratio of 40 miRNAs was significantly increased and that of 23 miRNAs was significantly decreased in gastric cancer compared to the adjacent normal tissues (Table II).

MiR-193a inhibits gastric cancer cell growth and motility. Among the candidates, we selected miR-193a for further study because the biological function of its 5p and 3p arms remains unclear in gastric cancer. As shown in Figure 1C, miR-193a-5p was significantly decreased in gastric cancer

Figure 1. Identification of arm selection preference of miRNA in gastric cancer. (A): Workflow for analyzing the changes in the arm selection preference of miRNA candidates in gastric cancer. The small RNA transcriptome profiles of gastric cancer (45 normal and 399 tumor tissues) were downloaded from The Cancer Gene Atlas. The arm selection preference change candidates were selected with a significantly increased (fold change >1.5; $p < 0.01$) or decreased (fold change <0.67; $p < 0.01$). (B): A correlation was observed in the miR-#5p/miR-#3p ratio of 261 miRNAs between gastric cancer and normal tissues. (C-D): The expression levels of miR-193a-5p and miR-193a-3p were examined in gastric cancer and corresponding adjacent normal tissue from 37 patients. (E): The ratio of miR-193a-5p/-3p was analyzed in gastric cancer and corresponding adjacent normal tissue from 37 patients. The expression levels of the miRNA were presented in transcripts per million and analyzed using Student's *t*-test ($p < 0.05$ was considered significant). FPKM, Fragments per kilobase million.

compared to the adjacent normal tissue ($p=0.04$). No differences were observed in the expression levels of miR-193a-3p between gastric cancer and adjacent normal tissue (Figure 1D). The miR-193a-5p/miR-193a-3p ratio was

significantly decreased in gastric cancer tissues compared to the corresponding normal tissues ($p=0.005$) (Figure 1E), indicating changes in the arm selection preference during the miRNA maturation process.

Table II. *miRNA candidates that showed changes in arm selection preferences in gastric cancer compared to normal tissues.*

Up-regulation of miR-#-5p:-#-3p ratio (tumor/normal)	Down-regulation of miR-#-5p:-#-3p ratio (tumor/normal)
hsa-let-7e, hsa-mir-7, hsa-mir-15a, hsa-mir-25, hsa-mir-27b, hsa-mir-29a, hsa-mir-29b, hsa-mir-30c, hsa-mir-31, hsa-mir-92a, hsa-mir-92b, hsa-mir-96, hsa-mir-105, hsa-mir-122, hsa-mir-130a, hsa-mir-135b, hsa-mir-182, hsa-mir-183, hsa-mir-188, hsa-mir-191, hsa-mir-196a, hsa-mir-196b, hsa-mir-337, hsa-mir-425, hsa-mir-431, hsa-mir-502, hsa-mir-509, hsa-mir-512, hsa-mir-517a, hsa-mir-517b, hsa-mir-519a, hsa-mir-550a, hsa-mir-654, hsa-mir-671, hsa-mir-675, hsa-mir-767, hsa-mir-877, hsa-mir-1228, hsa-mir-2116, hsa-mir-3679	hsa-mir-9, hsa-mir-10a, hsa-mir-10b, hsa-mir-20b, hsa-mir-26a, hsa-mir-29b, hsa-mir-99b, hsa-mir-100, hsa-mir-129, hsa-mir-193a, hsa-mir-154, hsa-mir-199a, hsa-mir-335, hsa-mir-369, hsa-mir-377, hsa-mir-423, hsa-mir-455, hsa-mir-485, hsa-mir-490, hsa-mir-491, hsa-mir-518a, hsa-mir-615, hsa-mir-1914

To investigate the biological function of the individual arms of miR-193a, miR-193a-5p and miR-193a-3p mimics were transfected into the AGS cells, respectively. Following transfection, the expressions of miR-193a-5p and -3p were significantly increased in the transfected cells compared to the scrambled controls (Figure 2A and B). Ectopic miR-193a-5p slightly suppressed the proliferation and colony formation in AGS cells, and miR-193a-3p expression strongly inhibited cell growth (Figure 2C–E). However, only miR-193a-3p significantly inhibited the cell invasion ability (Figures 2F and 2G). These results revealed that miR-193a-5p and -3p were generated from an identical pre-mir-193a; however, the arm selection preference differed significantly between gastric cancer and normal tissues. Furthermore, miR-193a-3p played crucial role in modulating the growth and invasion ability of gastric cancer cells.

Identification of target genes of miR-193a-3p in gastric cancer. We further identified putative target genes of miR-193a-3p using the TargetScan tool (release no. 7.0); we found that 76 putative protein-coding genes might be regulated by miR-193a-3p (Figure 3A). Our results showed that miR-193a-3p plays a tumor suppressive role by inhibiting gastric cancer cell growth and motility; therefore, indicated that the target genes of miR-193a-3p in gastric cancer might be oncogenes. By analyzing the TCGA database, 18 gene expression levels were significantly increased in gastric cancer compared to the adjacent normal tissues. Previous studies revealed that CCND1 and ETS1 were involved in accelerating gastric cancer cell growth and invasion ability (22, 23). Thus, CCND1 and ETS1 were selected for further study. Our data revealed that ectopic miR-193a-3p expression significantly suppressed CCND1 and ETS1 expression both in mRNA and protein levels

(Figures 3B and 3C). The reporter assay also showed that miR-193a-3p suppressed luciferase activity by directly targeting the 3'UTR of ETS1 (Figure 3D). CCND1 and ETS1 were significantly overexpressed in gastric cancer compared to normal tissue (Figures 4A and B), and their knockdown significantly suppressed gastric cancer cell colony formation, proliferation, and anchorage-independent growth, similar to miR-193a-3p overexpression in gastric cancer cells (Figures 4C–I). In summary, our results indicated that miR-193a-3p suppressed gastric cancer cell growth by directly targeting CCND1 and ETS1 expression.

Discussion

Gastric cancer is usually diagnosed at an advanced stage and is often accompanied with metastasis, leading to poor survival. miRNAs have been reported to play a crucial role in the tumorigenesis and progression of gastric cancer (5, 6). In particular, miR-193a has been shown to have differential expression in gastric cancer (24, 25). Huang *et al.* reported that Linc00152 could promote gastric cancer growth by sponging miR-193a-3p expression (26). However, the detailed role of miR-193a-5p and miR-193a-3p in gastric cancer remains unknown. The present study is the first to report that both miR-193a-5p and miR-193a-3p play a tumor suppressive role in gastric cancer.

The pre-miR-193a could generate both miR-193a-5p and miR-193a-3p. We found that the ratio of the miR-193a-5p/miR-193a-3p was significantly decreased in gastric cancer compared to the adjacent normal tissues. In addition, miR-193a-5p, but not miR-193a-3p, was significantly decreased in gastric cancer compared to the corresponding normal tissues. Studies have reported that mature miRNA prevents degradation by interacting with its target genes (16, 17).

Figure 2. Biological function of the miR-193a-5p and -3p arms was examined in AGS cells. (A-B): The expression levels of miR-193a-5p and miR-193a-3p in AGS cells with or without mimic transfection were examined by real-time PCR. (C-D): The colony formation assay was performed in the AGS cells after transfection with miR-193a-5p mimics, miR-193a-3p mimics, and a scramble control for 2 weeks. The cells were fixed and stained with crystal violet solution, and relative colony formation was quantified. (E) After miR-193a-5p or -3p mimic transfection, cell proliferation was measured using the CellTiter-Glo® One Solution assay at various time points (0, 1, 2, 3, and 4 days) and compared to the scramble control. (F-G): After miR-193a-5p or -3p mimic transfection for 12 h, the invasion ability of the cells was examined using the Transwell assay. The invading cells were stained with crystal violet solution and were enumerated by counting three fields under a phase-contrast microscope. NC, Negative control; ns, non-significant.

Figure 3. Identification of putative target genes of miR-193a-3p by bioinformatics and experimental approach. (A): The flowchart to identify putative target genes of miR-193a-3p by analyzing TargetScan tool and TCGA database. (B-C): The expression levels of CCND1 and ETS1 in AGS cells transfected or not with miR-193a-3p mimic were examined through real-time PCR and western blotting. (D): The luciferase reporter assay was examined in AGS cells with or without miR-193a-3p expression.

Therefore, the concept of TMMP could explain why the miR-193a-5p expression levels were decreased whereas those of miR-193a-3p were not. Taken together, these noteworthy phenomena might be due to higher number of targets of miR-193a-3p compared to miR-193a-5p in gastric cancer.

A pre-miR-193a could produce miR-193a-5p or miR-193a-3p; however, the biological function might be distinct depending on their target genes. Previous studies have revealed that both miR-193a-5p and miR-193a-3p suppressed lung cancer cell migration and invasion by the co-regulation of ERBB4/PIK3R3/mTOR/S6K2 signaling pathway activity (27). They were also reported to play important roles in osteosarcoma metastasis through the inhibition of Rab27B and SRR genes (28). In the present study, functional assay showed that both miR-193a-5p and miR-193a-3p could suppress gastric cancer cell growth, but only miR-193a-3p inhibited gastric cancer cell motility. These results are consistent with those of our previous study

that demonstrated that miR-193a-3p plays a crucial role in suppressing breast cancer growth and invasion (17).

Our data revealed that miR-193a-5p slightly suppressed gastric cancer cell growth, with no effect on gastric cancer invasion ability. Studies have shown that miR-193a-5p might play the opposite role in tumor development. miR-193a-5p plays the role of an oncogene in regulating cancer chemosensitivity by repressing TP73 and AP-2 α expression in squamous cell carcinoma, bone tumor, and bladder cancer (29-31). However, others studies have revealed that miR-193a-5p plays contrasting dual functions in suppressing cancer development. Lin *et al.* have reported that miR-193a-5p increased radiosensitivity and suppressed the tumorigenesis of esophageal squamous cell carcinoma by directly targeting the ERBB2 expression (32). In human endometrioid endometrial adenocarcinoma, miR-193a-5p suppressed cancer cell growth and migration by modulating the miR-193a-5p-YY1-APC axis (33). Our previous study revealed that miR-193a-5p

Figure 4. Biological function of CCND1 and ETS1 knockdown was examined in AGS cells. (A-B): The expression levels of CCND1 and ETS1 in gastric cancer tissues were analyzed in comparison to the corresponding adjacent normal tissues using the TCGA database. (C-D): The expression levels were examined in AGS cells transfected with small interfering RNA of CCND1 (si-CCND1), ETS1 (si-ETS1), or scramble control using western blotting. (E-F): The colony formation assay was performed in the AGS cells with si-CCND1, si-ETS1, or scramble control transfection for 2 weeks. The cells were fixed and stained with crystal violet solution, and relative colony formation was quantified. (G): After CCND1 or ETS1 knockdown, cell proliferation was measured using the CellTiter-Glo® One Solution assay at various time points (0 and 4 days) and was compared to the scramble control. (H-I): After CCND1 or ETS1 knockdown, the anchorage-dependent growth ability of the cells was examined using the soft agar assay. The colony was stained with iodinitrotetrazolium chloride solution and was enumerated by counting three fields under a phase-contrast microscope. FPKM, Fragments per kilobase million.

suppressed breast cancer cell growth but did not influence the migration ability by suppressing NLN expression (17). These inconsistent results may be due to differences in cancer type. In the present study, miR-193a-5p was found to play a tumor suppressive role by slightly inhibiting gastric cancer cell growth, but the detailed mechanism remains unclear. Until now, miR-193a-3p has frequently exhibited a tumor suppressive role in human cancers (27, 34-43). Numerous studies have shown that miR-193a-3p could directly target oncogene expression, including uPA, MCL1, PLAU, K-Ras, CCND1, SEPN1, and IL17RD genes (17, 34-36, 42, 44, 45). In this study, we identified ETS1 as a new target of miR-193a-3p. Our data indicated that miR-193a-3p-induced suppression of gastric cancer cell growth might be at least partially caused by silencing of ETS1 and CCND1 expression.

In conclusion, both miR-193-5P and miR-193a-3p exert tumor-suppressing roles in gastric cancer. In particular, miR-193a-3p plays a crucial role in inhibiting gastric cancer growth and invasion ability by targeting ETS1 and CCND1 expression. These findings provide new insight into understanding the molecular mechanism underlying gastric cancer progression.

Acknowledgements

This work was supported by grants from Kaohsiung Veterans General Hospital (VGHKS-106-014 and VGHKS107-013) and Zuoying Branch of Kaohsiung Armed Forces General Hospital (ZBH-106-14 and ZBH-107-17).

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Received April 12, 2018

Revised May 9, 2018

Accepted May 11, 2018