Emerging role of microRNAs in modulating endothelin-1 expression in gastric cancer

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Abstract. Endothelin-1 (ET-1) is a small 21-amino acid peptide that is known to exert diverse biological effects on a wide variety of tissues and cell types through its own receptors. The ET-1-ETRA axis is frequently dysfunctional in numerous types of carcinomas, and contributes to the promotion of cell growth and migration. microRNAs (miRNAs) are small non-coding RNAs that play a critical role in carcinogenesis through mRNA degradation or the translational inhibition of cancer-associated protein-coding genes. However, the role of ET-1 and the relationship between ET-1 and miRNAs in gastric cancer remain unknown. Results of the analysis of the database of The Cancer Genome Atlas (TCGA) revealed that ET-1 is significantly overexpressed in gastric cancer cells when compared with its expression in adjacent normal cells. Exogenous ET-1 significantly enhanced gastric cancer cell proliferation, implying that ET-1 plays an oncogenic role in gastric cancer carcinogenesis. Using a luciferase reporter assay we showed that 18 miRNA candidates had a significant silencing effect on ET-1 expression by up to 20% in HEK293T cells. Among them, 5 miRNAs (miR-1, miR-101, miR-125A, miR-144 and let-7c) were shown to be involved in ET-1 silencing through post-transcriptional modulation in gastric cancer. Our data also revealed that DNA hypermethylation contributes to the silenced miR-1 expression in gastric cancer cells. The ectopic expression of miR-1 significantly inhibited AGS cell proliferation by suppressing ET-1 expression. Overall, our study revealed that ET-1 overexpression may be due to DNA hypermethylation resulting in the silencing of miR-1 expression in gastric cancer cells. In addition, we identified several miRNAs as potential modulators for ET-1 in gastric cancer, which may be used as targets for gastric cancer therapy.

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Introduction

Endothelin (ET) was first identified as a vasoconstrictive 21-amino acid S-peptide derived from vascular endothelial cells. It is currently known to exert diverse biological effects on a wide variety of tissues and cell types through its own receptors (1,2). Three ETs (ET-1, ET-2 and ET-3), 2 G-protein-coupled receptors (ETRA and ETRB) and 2 ET-converting enzymes (ECE-1 and ECE-2) comprise the ET axis (2). The ET-1 gene contains 5 exons located on chromosome 6, and expresses a 212-amino acid precursor, preproendothelin-1 (PPET-1). PPET-1 is converted by protease to form an intermediate peptide consisting of 38 amino acids, big-ET-1, which is further cleaved by ECE-1 or ECE-2 to release the mature form of ET-1 (21 amino acids) (3,4).

An increase in ET-1 expression has been detected in various malignancies including lung, prostate, colorectal, liver, breast and ovarian cancers, as well as melanomas, and may contribute to cell growth, metastasis and angiogenesis, and suppression of apoptosis (4-11). ET-1 typically induces proliferation through the ETRA receptor, and decreased ETRB expression has frequently been implicated in the pathogenesis of esophageal squamous cell carcinoma and nasopharyngeal carcinoma, as well as prostate and ovarian cancers (12). The promoter regulatory region of the ETRB gene contains a 5' CpG island which is commonly (70%) methylated, suggesting that ETRB down-regulation occurs through DNA hypermethylation in human

carcinomas (13-17). In gastric cancer, a high level of ET-1 has been detected in plasma, which may be a reliable marker for predicting recurrence (18,19). Stow *et al* (20) summarized previous studies and revealed that ET-1 transcription can be enhanced using numerous physical and chemical stimuli such as phorbol esters, thrombin, angiotensin II and insulin, by activating signaling transduction pathways including the protein kinase C (PKC) and JUN-terminal kinase (JNK) pathways. In addition, GATA family transcription factors, β-catenin, transforming growth factor β (TGFβ) and hypoxia-inducible factor-1 (HIF-1) can enhance ET-1 transcriptional activity by modulating its promoter (8,12). Therefore, unbalanced stimulators and impaired signaling that frequently lead to ET-1 overexpression trigger the activation of the ET axis, and promote cancer cell progression through autocrine and paracrine signaling. However, the detailed functions and regulatory mechanisms of ET-1 in gastric cancer remain unknown. The number of studies has recently been increasing regarding small non-coding short RNA microRNAs (miRNAs) in cancer development through post-transcriptional regulation of the expression of oncogenes or tumor-suppressor genes. miRNAs are deregulated in numerous disease states, particularly in cancer, making them novel molecular indicators that are critical for early diagnosis, prognosis, and therapy. Previous studies have demonstrated several instances of abnormal miRNA expression in gastric cancer cells compared with adjacent normal cells (21,22). These dysfunctional miRNAs may disrupt the balance of their target genes, resulting in the downregulation of tumor-suppressor genes and oncogene overexpression in gastric cancer.

Previous studies have revealed that miRNAs play a critical role in modulating ET-1 expression by targeting the 3'untranslated region (3'UTR), including miR-1, miR-125a, miR-125b, miR-155 and miR-199 (23-28). To date, the biological function and regulating mechanisms of ET-1 by miRNAs in gastric cancer remain unknown. In the present study, we demonstrated that ET-1 plays an oncogenic role in gastric cancer growth, and identified several potential target sites for miRNA candidates harbored within the 3'UTR of ET-1 using a bioinformatics approach. We identified 5 dysfunctional miRNAs that may contribute to ET-1 overexpression in gastric cancer, to provide a potential approach and novel targets for therapy.

Materials and methods

Cell line and nuclear acid extraction. Five human gastric cancer cell lines, AGS, AZ-521, HR, NUGC and TSGH, were obtained from the American Type Culture Collection, and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA). After 5-Aza-dC (10 μM) treatment for 4 days, RNA was extracted using TRIzol reagent (Invitrogen), according to the manufacturer's protocol. After RNA extraction, the interface was subjected to DNA extraction with a back extraction buffer (4 M guanidine thiocyanate, 50 mM sodium citrate and 1 M Tris). Genomic DNA was then precipitated with EtOH.

Constructed reporter of ET-1 and the miRNA expression vector. ET-1 3'UTR was amplified from AGS cDNA by using the specific primer. The amplified ET-1 3'UTR was cloned into the pmiR-RB-Report vector, between the *Xho*I and *Not*I restriction sites. The primary miRNA transcripts were amplified from the genomic DNA of AGS cells by using miR-specific forward and reverse primers. The amplified PCR products were then cloned into the pLKO vector, between the *Age*I and *Eco*RI restriction sites. All constructs were further confirmed through direct sequencing. The individual primers used in the present study are shown in Table I.

Luciferase reporter assay. HEK293T cells ($2.5x10^4$ cells/well) were seeded in a 24-well plate and cotransfected with 0.3 μg of either the control vector or various miRNA constructs in conjunction with 0.1 μg of the ET-1-3'UTR reporter construct (pmiR-RB-REPORT™; RiboBio) by using the Lipofectamine 2000 reagent (Invitrogen). After 48 h, the luciferase activity was measured using the Dual-Glo Luciferase Assay System kit (Promega, Madison, WI, USA). Luciferase readings were corrected for background, and firefly luciferase values were normalized to *Renilla* values to control for transfection efficiency. All of the assays were performed in triplicate in 3 independent experiments.

Methylation status of CpG islands. The genomic DNA of 5 gastric cancer cell lines, and normal and tumor tissue pairs from 50 gastric cancer patients were subjected to bisulfite conversion using the EZ DNA Methylation-Gold kit (Zymo Research Corporation, Orange, CA, USA). The bisulfite conversion reaction was conducted at 98˚C for 10 min, and then at 64˚C for 2.5 h, with a final incubation at 4˚C for up to 20 h in a PCR thermocycler. Then, the modified genomic DNA was used for the methylation analysis of CpG25 and CpG81 of the mir-1-1 upstream using the specific methylation primers. The PCR conditions were as follows: 94˚C for 10 min, followed by 35 cycles of 94˚C for 1 min, 60˚C for 1 min, and 72˚C for 30 sec, with a final extension at 72˚C for 10 min using a PCR thermocycler and HotStart *Taq* DNA polymerase (Qiagen, Hilden, Germany). The methylation status of the genomic DNA of individual samples was also examined with the *Bst*UI digestion (New England Biolabs, Beverly, MA, USA). The digested PCR fragments were then separated on 2% agarose gel. Detailed information is provided in a previous study (29). The individual primers used in the present study were as follows: CpG25-F, 5'-GGAGGGGTAGGATAGTAGTTTG AGT-3' and CpG25-R, 5'-AAAAAAACCTAACCTAAAAAA CCAAAA-3'; CpG81-F, 5'-GGTGAGTTTTGTTTAGT TTATTATTAT-3' and CpG81-R, 5'-ATCAAAATTCCTA CCTCCCAACTA-3'.

Stem-loop RT-PCR of miR-1. Total RNA (2 μg) was reverse-transcribed with miR-1-specific stem-loop RT primers using SuperScript III Reverse Transcriptase (Invitrogen). The reaction was performed under the following incubation conditions: 30 min at 16˚C, followed by 20˚C for 30 sec, 42˚C for 30 sec and 50˚C for 1 sec, totaling 50 cycles. The enzyme was subsequently inactivated by incubation at 85˚C for 5 min. Afterward, cDNA was diluted 10X for subsequent real-time PCR analysis using the miR-1-GSF primer. Gene expression was detected using a SYBR-Green I assay (Applied Biosystems, Foster City, CA, USA). The U6 expression was used as the internal control,

Table I. Primer sequences.

Constructed primers	Sequences
ET-1-3'UTR-F	5'-CCGCTCGAGCAGACCTTCGGGGCCTGTCTGAAG-3'
ET-1-3'UTR-R	5'-GAATGCGGCCGCACAGTAAGGAAAAAAATATTTATTTTC-3'
miR-1-1-F	5'-TCTACCGGTGGACACCAGGCAGCAGTGGC-3'
miR-1-1-R	5'-TCTGAATTCACAATGCTGGCGGGGACACG-3'
let-7b-F	5'-TCTACCGGTGGGCCTCTGCCTGTGGAGGA-3'
let-7b-R	5'-TCTGAATTCTCACTGAGGTAGGGGGCGGC-3'
let-7c-F	5'-TCTACCGGTTGGCAGGTTAGATGGTCAGAAGACA-3'
let-7c-R	5'-ACCTTCTTGCACACAAATTGGCTCA-3'
miR-33a-F	5'-TCTACCGGTCCCATAGCCTCTGTAAGCCC-3'
miR-33a-R	5'-TCTGAATTCGCTAAGGACATGTTCCCCGT-3'
miR-101-F	5'-TCTACCGGTTGCCTCCTCACGTCTCCAACCA-3'
miR-101-R	5'-TCTGAATTCTGGCTGCACCAACAACTACCCC-3'
miR-125a-F	5'-TCTACCGGTTTCTGTCCTTGTCCCTGCATC-3'
miR-125a-R	5'-TCTGAATTCCCATCGTGTGGGTCTCAAGG-3'
miR-125b-F	5'-TCTACCGGTTGCGCCCCCAGATACTGCGT-3'
miR-125b-R	5'-TCTGAATTCGGGGCATGCTGGGACTTCAGC-3'
miR-134-F	5'-TCTACCGGTCGGGCCATGGACAATGCGCT-3'
miR-134-R	5'-TCTGAATTCAGGGGCTGCCAAGGCTGACT-3'
miR-135a-F	5'-TCTACCGGTTCTTGTTTCCCGGTCCTTGT-3'
miR-135a-R	5'-TCTGAATTCACCCGACTGGTGGGCTATTA-3'
miR-135b-F	5'-TCTACCGGTTTTATGGCCAGGAAGCCACC-3'
miR-135b-R	5'-TCTGAATTCCAGCACACCCTGAAGGTCTC-3'
miR-141-F	5'-TCTACCGGTGGACACAATGGGCCCCAGCC-3'
miR-141-R	5'-TCTGAATTCAAAGCAGACGTCGCAGCCCC-3'
miR-144-F	5'-TCTACCGGTACACTGGCCCTGGGTCCCTA-3'
miR-144-R	5'-TCTGAATTCTGCCCTGGCAGTCAGTAGGTT-3'
miR-203-F	5'-TCTACCGGTGTCGGGGGCTCCTCTCTCCG-3'
miR-203-R	5'-TCTGAATTCCACCGCCAGTTCCTCGCTGG-3'
miR-224-F	5'-TCTACCGGTGCAGAGGGCTGGGCTACCTT-3'
miR-224-R	5'-TCTGAATTCCCCAGGGCCCAACTGGAAGAGT-3'
miR-340-F	5'-TCTACCGGTTCCAGCTTGAGTCTTCAAGAG-3'
miR-340-R	5'-TCTGAATTCAGAGTTGTGATCAGTAAATTAGA-3'
miR-379-F	5'-TCTACCGGTGCCTGCTTCCAATGCCAAAT-3'
miR-379-R	5'-TCTGAATTCGCCCAAGTTGCATCACTTCC-3'
miR-410-F	5'-TCTACCGGTGCCCTTTTGAGGGTAGGAGC-3'
miR-410-R	5'-TCTGAATTCAATGATTCAATGGCGGGGGT-3'
miR-425-F	5'-TCTACCGGTGTGCCCCTGACCCCCAGACA-3'
miR-425-R	5'-TCTGAATTCAGCAGGGAAACCCAGGGGCA-3'
miR-494-F	5'-TCTACCGGTACCGTCAGGAAAGCTCCAAT-3'
miR-494-R	5'-TCTGAATTCTCAGGAACAGGAAGTGCCTC-3'
miR-495-F	5'-TCTACCGGTCGCCTCTGCTCAGTGTCAGCC-3'
miR-495-R	5'-TCTGAATTCTCAGGGTCCCGTCGGGGATG-3'
miR-590-F	5'-TCTACCGGTGAACGTCAGCACCCTCCCCCCA-3'
miR-590-R	5'-TCTGAATTCTTGAGCGCCAGTGGCCAAGC-3'
miR-873-F	5'-TCTACCGGTGTGACCAGTGTCTGGGATGC-3'
miR-873-R	5'-TCTGAATTCCCTTGGTGGGATTCAACACCT-3'
Gene-specific primer	
miR-1-RT	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGATACATAC-3'
miR-1-GSF	5'-CGGCGGTGGAATGTAAAGAAGT-3'

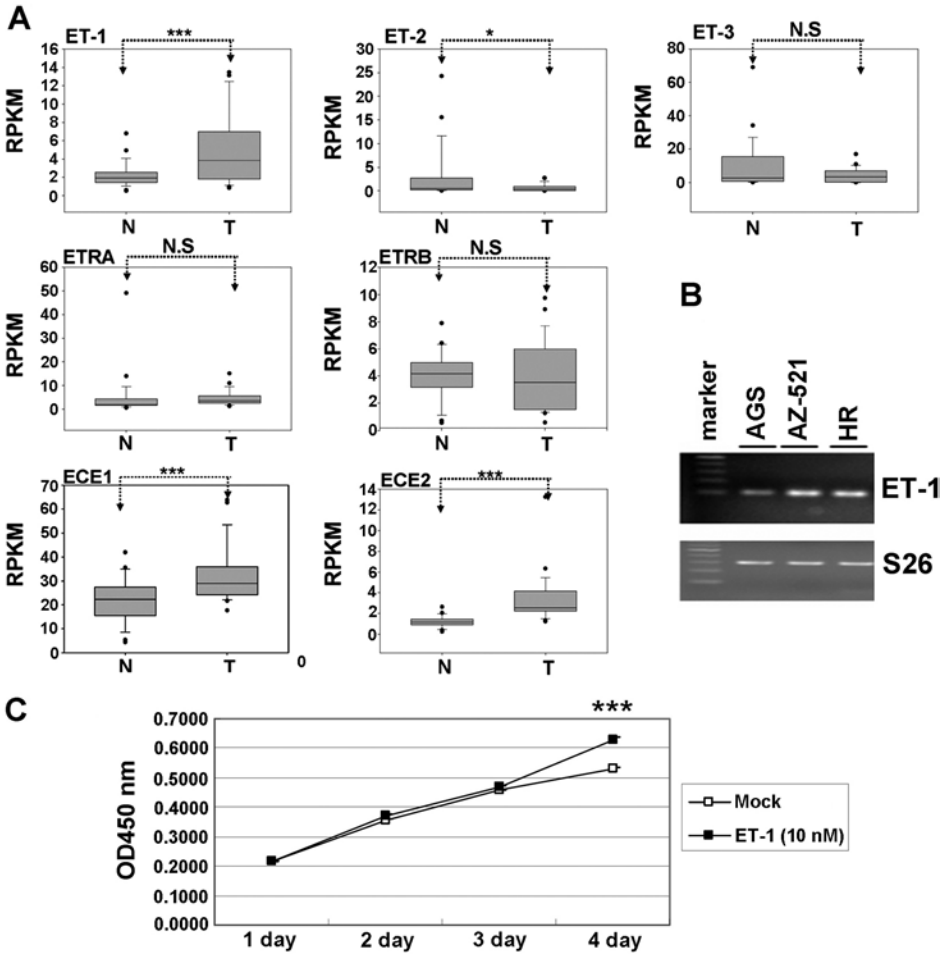

Figure 1. Expression levels and function of the endothelin axis in gastric cancer cell lines. (A) The expression levels of ET-1, ET-2, ET-3, ETRA, ETRB, ECE-1 and ECE-2 were examined between gastric cancer and adjacent normal from 29 patients by analyzing the TCGA database. The expression levels of the genes were presented in reads per kilobase per million reads (RPKM). The expression level between the tumor and normal cells was evaluated by conducting paired t-tests (P<0.05 was considered significant; NS, non-significant; *P<0.05, ** P<0.01, ***P<0.001). (B) The mRNA level of ET-1 was assessed in 3 human gastric cancer cell lines. (C) Exogenous ET-1 stimulation enhanced AGS cell proliferation. ET-1, endothelin-1; ETRA and ETRB, G-protein-coupled receptors; ECE-1 and ECE-2, ET-converting enzymes; TCGA, The Cancer Genome Atlas.

and the expression levels of miR-1 were normalized to that of U6 ($\Delta Ct = Ct_{miR-1} - Ct_{U6}$).

Ectopic expression of miRNA candidates. Stable or transient gastric cell cultures expressing miR-1 candidates were generated using the transfected gastric cells with pLKO-pre-miRNA by Lipofectamine 2000. After 24 h of transfection, the stably expressed miRNA cells were generated by puromycin selection for 14 days. The shLuc targeting the luciferase gene provided puromycin resistance as the control. The expression levels of miRNAs were confirmed using real-time PCR.

Expression levels of ET axis genes and miRNAs were analyzed using The Cancer Genome Atlas (TCGA) data. The TCGA project collects RNA transcriptome and small RNA transcriptome data, which are obtained using the next-generation sequencing approach from various types of human cancers. For the present study, we downloaded all level-3 expression data for gastric cancer from the TCGA data portal (https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm). We used 29 tumor samples and 29 normal samples to analyze the expression levels of each miRNA and ET axis-related gene.

Cell proliferation assay. For cell proliferation analysis, we seeded 3,000 living AGS cells or AGS cells stably expressing miR-1 in 96-well plates. After exogenous ET-1 stimulation, we determined cell growth at 0, 1, 2, 3 and 4 days using WST-1 (Roche, Mannhein, Germany). All experiments were repeated 3 times.

Results

ET-1 is upregulated in gastric cancer. We first assessed the expression pattern of the ET axis genes (ET-1, ET-2, ET-2, ETRA, ETRB, ECE-1 and ECE-2) in gastric cancer by analyzing data from the database of TCGA. We obtained RNA transcriptome data on cancer tissues and the corresponding adjacent normal tissues from information on 29 gastric cancer patients. As shown in Fig. 1A, the expression levels of ET-1, ECE-1 and ECE-2 were significantly increased in gastric cancer compared to corresponding normal tissues (P<0.001). Conversely, ET-2 expression was decreased significantly in gastric cancer (P<0.05). We further examined the expression levels of ET-1 in 3 gastric cancer cell lines using the RT-PCR approach. As shown in Fig. 1B, we detected a greater abun-

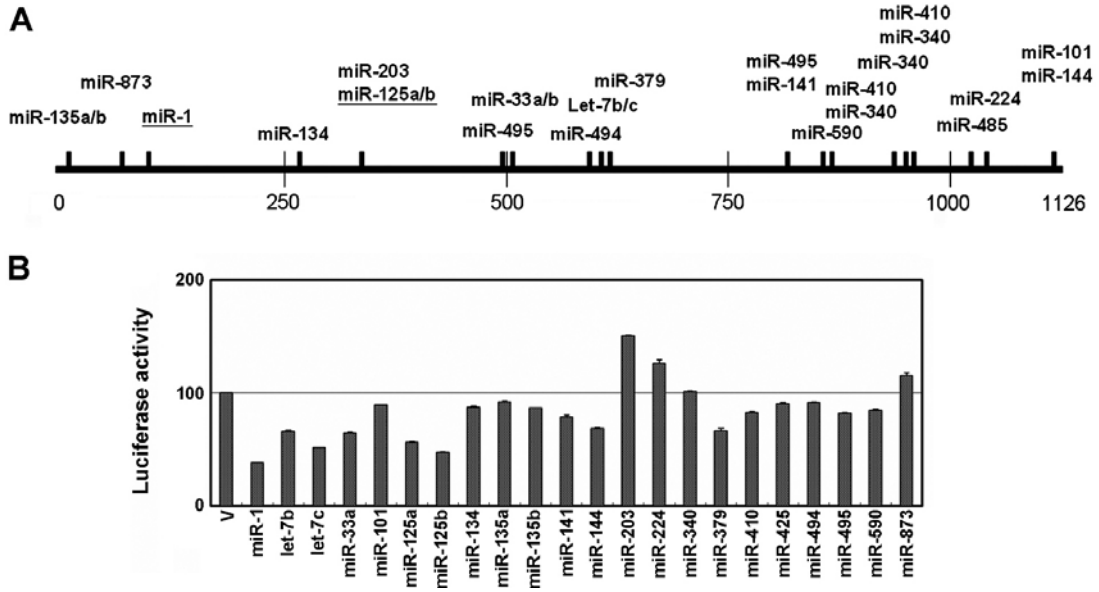

Figure 2. Putative microRNA candidates mediate ET-1 expression by targeting the 3'UTR of ET-1 in gastric cancer. (A) Schematic diagram of the luciferase constructs containing 3'UTR sequences of ET-1 (1,126 bp), which were cloned downstream of the luciferase gene. Predicted miRNA target sites within the 3'UTR of ET-1 through microRNA.org prediction program. The underlined miRNAs indicate that these candidates have been reported to target the 3'UTR sequence of ET-1. (B) ET-1 3'UTR was cloned into the pmiR-RB-Report vector and then were cotransfected with various miRNA constructs into HEK293T cells. Luciferase assay was conducted after 48 h cotransfection in HEK293T cells using the Dual-Glo Luciferase Reporter Assay System kit. ET-1, endothelin-1; 3'UTR, 3' untranslated region.

dance of ET-1 RNA in the AZ-521 and HR cells, and low expression in AGS cells. To investigate the putative function of ET-1 on gastric cancer, we further treated AGS cells with ET-1 (10 nM) for 4 days. We evaluated the potential influence of ET-1 on gastric cancer cell proliferation through WST-1 for 96 h. As shown in Fig. 1C, cell proliferation in the ET-1 treatment groups was significantly increased compared with that in the control groups. These results demonstrate that ET-1 plays an oncogenic role in promoting the proliferation of gastric cancer cells. However, the mechanism that leads to ET-1 dysregulation in gastric cancer remains unclear. To further investigate the underlying mechanisms of ET-1 dysfunction in gastric cancer, we focused on the post-transcriptional regulation of ET-1 expression by miRNAs during the progression of gastric cancer.

Discovery of putative miRNAs that regulate ET-1 genes. Using a prediction program available on microRNA.org, we predicted that several miRNA candidates could suppress ET-1 expression by targeting its 3'UTR sequence. Among them, we selected 22 miRNA candidates, and constructed their pre-miRNA sequence into a pKLO expression vector (Fig. 2A). In addition, we cloned the full length of the 3'UTR (i.e., 1,126 bp) of ET-1 into the pmiR-RB-Report vector. Due to the fact that the HEK293T cells have a high transfection efficiency, we conducted a luciferase assay using HEK293T cells. As shown in Fig. 2B, miR-1, let-7c and miR-125b substantially inhibited luciferase activity by >50% compared to the control group after transfection for 48 h. Fifteen miRNA candidates slightly suppressed the luciferase activity, including let-7b, miR-33a, miR-101, miR-125a, miR-134, miR-135a/b, miR-141, miR-144, miR-379, miR-410, miR-425, miR-494, miR-495 and miR-590. Consistent with previous studies, our data indicated that miR-1 and miR-125a/b both suppressed ET-1 expression through post-transcriptional regulation. After using a reporter assay, we found that 18 miRNA candidates may be involved in ET-1 post-translational regulation through the targeting of its 3'UTR sequence.

Expression level of miRNA candidates from the TCGA database. After completing the reporter assay, we selected 18 miRNA candidates to further examine their expression levels in gastric cancer by analyzing data from the TCGA database. As shown in Fig. 3A, we identified 6 miRNAs (miR-134, miR-135b, miR-141, miR-425, miR-494 and miR-590) and 5 miRNAs (miR-1, let-7c, miR-101, miR-125a and miR-144) that were significantly upregulated and downregulated, respectively, in gastric cancer. Since the expression level of ET-1 was upregulated in the gastric cancer cells compared with the corresponding normal cells, miRNA candidate expression should decrease in gastric cancer. According to their expression levels in gastric cancer, we selected 6 miRNAs (miR-1, miR-101, miR-125a/b, miR-144 and let-7c) for further confirmation in the AGS cells. These miRNA candidates were ectopically expressed in the AGS cells, and let-7c, miR-1, miR-101, miR-125a/b and miR-144 were found to significantly suppress luciferase activity (Fig. 3B). In addition, we found that the endogenous protein levels of ET-1 were inhibited by let-7c, miR-1, miR-101, miR-125a and miR-144 overexpression (Fig. 3C).

DNA hypermethylation silences miR-1 expression in gastric cancer. Among the listed miRNAs, we selected miR-1 to further examine the biological function and the regulatory mechanisms in gastric cancer. The expression of miR-1 originates from 2 paralogous genes, mir-1-1 and mir-1-2, located on chromosomes 20 and 18, respectively. As shown in Fig. 4A, 4 CpG islands are located at the putative transcription start

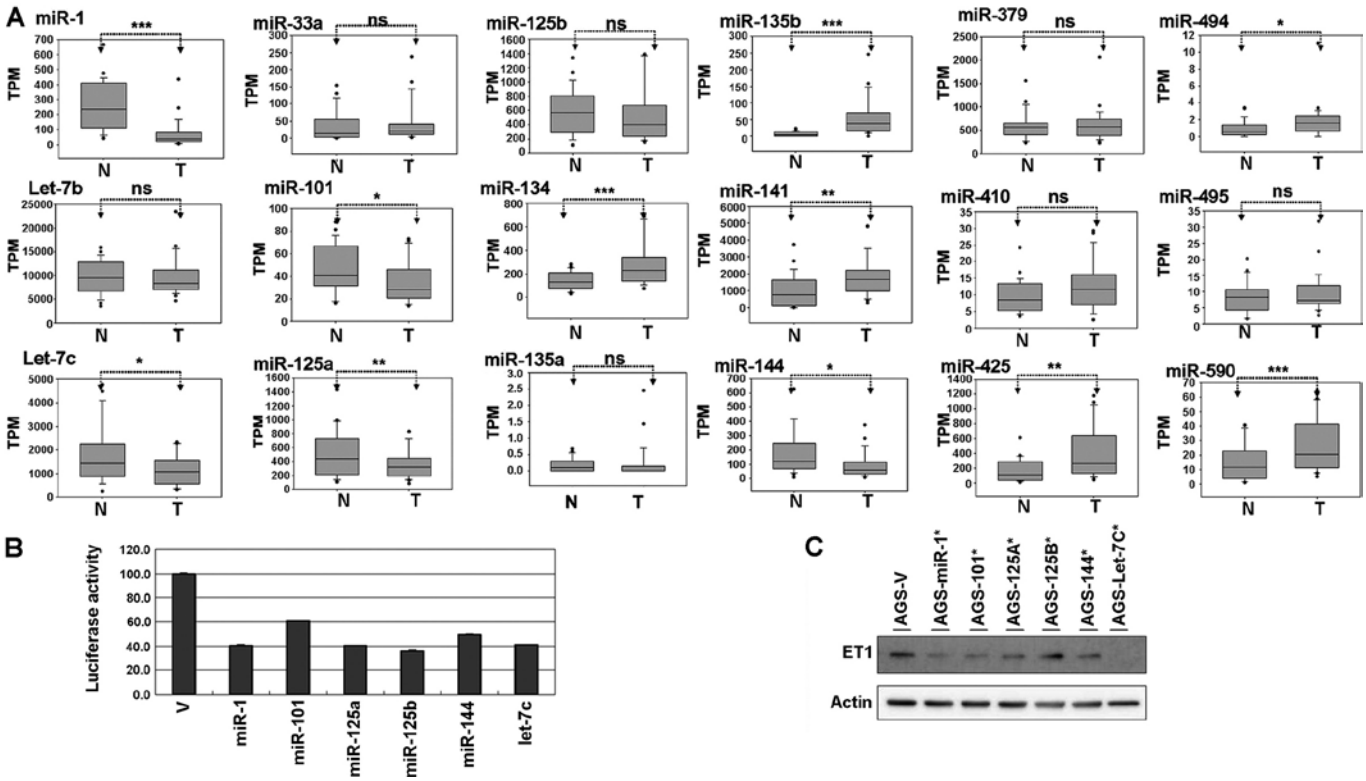

Figure 3. Suppression of ET-1 expression by miRNAs in gastric cancer cells. (A) Expression levels of the miRNA candidates in gastric cancer. Comparison of the miRNA expression levels between the tumor (T) and corresponding normal (N) tissues obtained from 29 gastric cancer patients, which were analyzed using the TCGA dataset. The expression levels of the miRNAs are presented in transcripts per million (TPM). The expression level between tumor and normal cells was evaluated by conducting paired t-tests ($P<0.05$ was considered significant; NS, non-significant, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$). (B) The reporter of ET-1 3'UTR was cotransfected with various miRNA constructs [vector (V), miR-1, miR-101, miR-125a/b, miR-144 and let-7c] into AGS cells. Luciferase assay was conducted after 48 h of cotransfection in AGS cells using the Dual-Glo Luciferase Reporter Assay System kit. (C) After 48 h transfection, the endogenous protein levels of ET-1 were examined by western blot analysis. ET-1, endothelin-1; 3'UTR, 3' untranslated region.

sites of the miR-1-1 loci, according to the UCSC database. We analyzed the methylation statuses of the CpG25 and CpG81 regions in 5 human gastric cancer cell lines by using a COBRA approach. As shown in Fig. 4B, the CpG81 regions were completely methylated in the AGS and AZ-521 cells, yet unmethylated in the HR cells. After treatment for 4 days with 5-Aza-dC, the expression levels of miR-1 were restored in the AGS, AZ-521 and HR cells (Fig. 4C). These data indicate that DNA methylation contributes to the modulation of miR-1 transcriptional activity in gastric cancer cell lines.

By analyzing TCGA data, we found that the expression levels of miR-1 were significantly decreased in the gastric cancer cells compared with the corresponding adjacent normal cells (Fig. 3A). Therefore, we further examined the methylation status of the CpG81 regions in 50 primary gastric cancers and the corresponding adjacent control, and observed a high frequence of DNA hypermethylation in gastric cancer (26 of 50). These results indicated that DNA hypermethylation leads to low expression levels of miR-1 in gastric cancer (Fig. 4D).

miR-1 suppresses gastric cancer cell growth. The ectopic expression of miR-1 in AGS cells increased mature miR-1 expression by ~250-fold (Fig. 5A). The growth of AGS cells with miR-1 overexpression was substantially inhibited compared with those transfected with the vector (Fig. 5B). Furthermore, the clonogenic survival of AGS cells also decreased after ectopic expression of miR-1 (Fig. 5C). Overall, our results imply that ET-1 overexpression may be due to the silencing of miR-1 expression by DNA hypermethylation in gastric cancer. In addition, we identified several miRNA candidates that could modulate the ET-1 oncogenic function, and could also act as potential therapeutic targets for therapy in the future.

Discussion

Dysfunction of the endothelin (ET) axis is known to be a feature of human cancer, including lung, prostate, colorectal, liver, breast, ovarian and oral cancer as well as melanomas. Abnormal activation of the ET axis leads to promotion of cell growth, metastasis and angiogenesis, and suppression of apoptosis (4-11). By analyzing TCGA data, we found that the expression levels of ET-1, ECE-1 and ECE-2 were significant increased in the gastric cancer compared to the corresponding normal tissues (Fig. 1). A previous study reported that the plasma big-ET-1 level in gastric cancer was significantly higher than that in the control (30). These findings imply that the expression level of ET-1 is increased in gastric cancer. Although ECE-1 (31) and ECE-2 were found to be upregulated in a number of cancers, including prostate, ovarian and oral cancer, thyroid carcinoma, and breast cancer (32-35), the expression of ECE-1 and ECE-2 was not reported in gastric

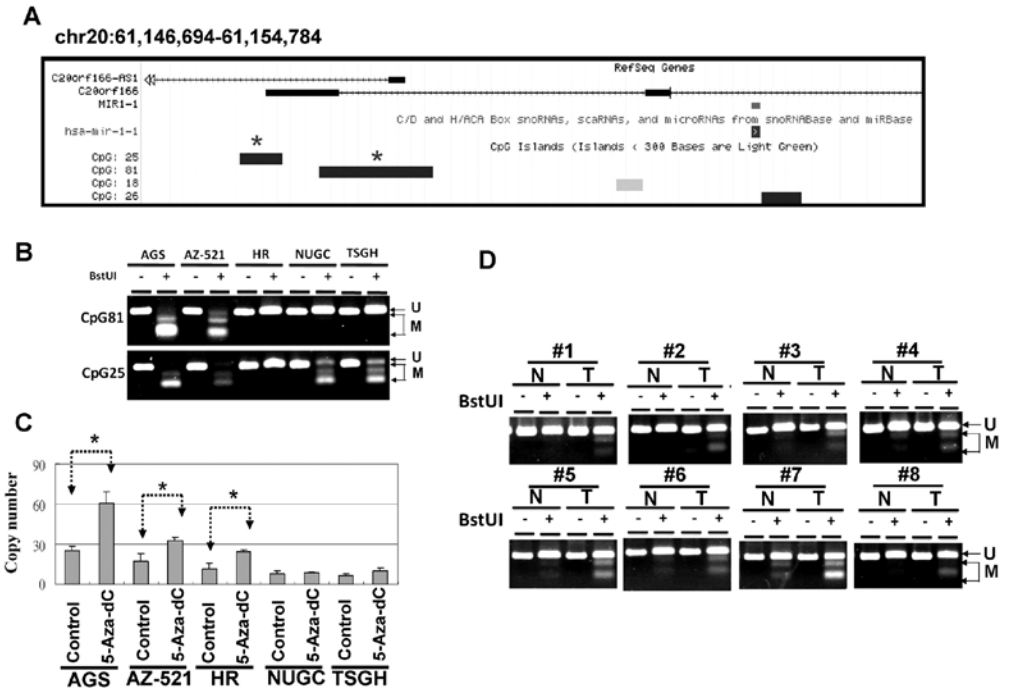

Figure 4. Expression of miR-1-1 is regulated by DNA methylation in gastric cancer. (A) Schematic representation of the locations of the mir-1-1 genes. Expressed sequence tag (EST) transcripts were identified from the UCSC website (http://genome.ucsc.edu/). Asterisks indicate the analyzed CpG islands (CpG25 and CpG81). (B) DNA methylation status of CpG25 and CpG81 of the mir-1-1 upstream region in 5 gastric cancer cell lines was examined by COBRA approach. M and U indicate the presence of methylated CpGs and unmethylated CpGs, respectively. (C) Real-time PCR analysis of miR-1 in 5 gastric cancer cell lines before and after treatment with 10 μM 5-Aza-dC; U6 expression was used as an internal control. Gene expression was calculated relative to that of an internal control (ΔCt). The number of copies of miR-1 was calculated using the standard equation 10,000 x ($2^{-\Delta Ct}$). (D) DNA methylation status of CpG81 of the mir-1-1 upstream region in gastric cancers examined by COBRA approach in normal (N) and tumor (T) tissue pairs from 50 gastric cancer patients. Eight representative cases are presented. M and U indicate the presence of methylated and unmethylated CpGs, respectively.

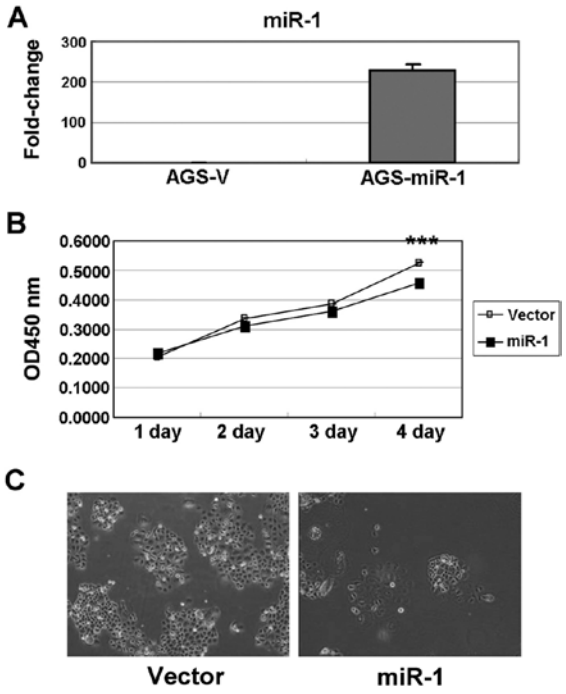

Figure 5. Ectopically overexpressed miR-1 inhibits cell growth. (A) Overexpression of miR-1 by Lipofectamine 2000 transfected into AGS cells and the expression level of miR-1 were examined by stem-loop RT-PCR. (B and C) Overexpression of miR-1 suppressed cell proliferation in AGS cells. Cells were transfected with either control pLKO-shLuc (as control) or pLKO-miR-1 for 24 h, which was followed by puromycin selection. Cell proliferation was examined by WST-1, and the colony formation was observed by microscopy.

cancer. Previous studies found that, in human cancer, ET-2 and ET-3 expression levels are silenced by DNA hypermethylation of their promoter regions (36,37). In the present study, we observed that the expression level of ET-2 was significant decreased in gastric cancer. However, what mechanism is involved in the loss of expression in gastric cancer remains unclear. These studies support our finding that the ET-1 axis is overexpressed, and that ET-2 is downregulated in gastric cancer.

In the past decade, miRNAs have emerged as playing important roles via modulation of the cell cycle, cell growth and cell motility in gastric cancer progression (21,22). In the present study, we identified 15 miRNAs that may modulate ET-1 expression by targeting 3'UTR. Among them, 6 miRNAs (miR-134, miR-135b, miR-141, miR-425, miR-494 and miR-590) and 5 miRNAs (miR-1, let-7c, miR-101, miR-125a and miR-144) showed significant upregulation and downregulation, respectively, in gastric cancer. Consistent with our findings, previous studies have revealed that miR-135b and miR-425 act as oncomiRs involved in the tumorigenesis of gastric cancer (38-40). Furthermore, miR-125a/b, miR-144 and miR-101 have been reported to be significantly decreased and display a tumor-suppressive function by silencing their oncongenic targets in gastric cancer. Here, we demonstrated that miR-125a/b, miR-101 and miR-144 suppressed ET1 expression by targeting the 3'UTR in gastric cancer. In addition, previous studies have reported that miR-1, miR-125a/b, miR-155 and miR-199 suppress ET-1 expression by targeting 3'UTR (23-28). In numerous cancers, miR-1 performs a

tumor-suppressive function by suppressing tumor cell growth and migration in prostate cancer, hepatocellular, bladder and esophageal squamous cell carcinoma, and lung cancer (29,41-48). Furthermore, hypermethylation of the promoter region of mir-1-1 resulted in low expression in hepatocellular carcinoma and colorectal cancer (29,48-50). In the present study, we also demonstrated that miR-1 expression levels were suppressed by DNA methylation and that ectopic miR-1 expression led to the suppression of gastric cancer cell growth. Collectively, our results imply that ET-1 overexpression may be due to DNA hypermethylation resulting in the silencing of miR-1 expression in gastric cancer.

In solid tumors, the ET-axis pathway plays a critical oncogenic role in facilitating cancer growth and metastasis. Various investigations have demonstrated that the ET-1-ETRA axis is a potential therapeutic target for cancer therapy. To date, several drugs have been designed as ETRA antagonists for anticancer growth and metastasis. The ET-1 is a short intercellular peptide derived from numerous tumor cells, and has emerged as a potential therapeutic target or biomarker for human cancer. In the present study, our identification of the post-translational regulation of ET-1 may lead to novel insights into the function of ET-1 in gastric cancer, and may provide a potential therapeutic target.

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