miR-125a regulates angiogenesis of gastric cancer by targeting vascular endothelial growth factor A

JUN DAI1, JINYU WANG2, LILI YANG1, YING XIAO1 and QIURONG RUAN1

1Institute of Pathology of Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030; 2Department of Pediatrics, The Second Affiliated Hospital, Zhejiang University College of Medicine, Hangzhou 310009, P.R. China

Received June 23, 2015; Accepted August 17, 2015

DOI: 10.3892/ijo.2015.3171

miRNAs are a class of small non-coding single-stranded RNAs. They combine with 3'- or 5'-untranslated region (UTR) of mRNAs in specific target genes to inhibit mRNAs translation or directly degrade their specific mRNAs, involving in regulating a variety of physiological and pathological processes such as cell proliferation, differentiation, apoptosis and metabolism (2-5). miR-125a is located in 19q13, which is frequently downregulated in several human cancers, including breast cancer (6), ovarian cancer (7), lung cancer (8) and medulloblastoma (9). Low expression of miR-125a was associated with the malignant potential indicators of enhanced GC such as tumor size and tumor invasion (10). Survival analysis indicated that low expression of miR-125a was an independent prognostic factor for GC patients, which may be related to the target gene Her-2 (11). However, the mechanism of miR-125a underlying GC is still unclear.

VEGF-A is considered to be the most potent angiogenic factors, which exerts its effect by activating vascular endothelial growth factor receptor 2 (VEGFR-2). During angiogenesis, VEGF-A binds to VEGFR-2 and activates multiple downstream pathways via signaling intermediates, such as PI3K/Akt (12-16). As a result, VEGF signaling may promote endothelial cell (EC) proliferation, survival, migration, filopodial extension and chemotaxis (17-19). VEGF-A plays an important role in gastric cancer, not only enhancing the malignant potential of tumor cells but also promoting the angiogenesis in the tumors (20-22). Moreover, expression level of VEGF-A in gastric cancer can directly affect the survival of patients (23).

The aim of the present study was to investigate the role of miR-125a in gastric cancer and the mechanism underlying it. We confirmed that miR-125a can function as a crucial tumor suppressor in human gastric cancer. The results showed that miR-125a decreased significantly in gastric cancer and was negatively correlated with the expression of VEGF-A, suggesting that miR-125a may inhibit angiogenesis of gastric cancer by downregulating VEGF-A.

Materials and methods

Patients. Consecutive series of 73 cases of gastric cancer and 20 cases of normal gastric tissues specimens were collected from Institute of Pathology of Tongji Hospital, Huazhong
University of Science and Technology in 2009. Patients with a previous history of another primary tumor, or those who had previously received chemotherapy and/or radiotherapy were excluded from the study. As to the diagnosis of gastric cancer, tumors were analyzed and discussed by two pathologists, and then a definite diagnosis was made. The age, sex, tumor size, lymph node (LN) metastasis and clinical stage of the patients were recorded.

**Cell culture.** Cell lines GES-1, AGS, SGC7901, BCG-823 and HUVEC were purchased from China Center for type culture collection in Wuhan. All cells were cultured with RPMI-1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS) (Gibco), while instructions were followed additionally under special circumstances.

**Cell transfection.** AGS cells were grown in 6-well plates to reach a cell density of 60% the next day. Lipofectamine 2000 (Invitrogen, USA) was mixed with miR-125a mimics and miR-125a inhibitors (GenePharma, China) respectively for 10 min. After washing cells twice with serum-Free-MEM medium, appropriate amount of opti-MEM was added to each well, then the above mixture was added to the medium dropwise to reach a concentration of 40 nM. The cells were incubated at 37˚C for 6 h, then the mixture was replaced with medium containing 10% FBS.

**QRT-PCR.** For miRNAs, in vivo, formalin-fixed paraffin-embedded tissue (FFPET) samples were cut into 10-µm thin slices, with a total thickness of 40 µm. miRNAs from FFPET embedded tissue (FFPET) samples were cut into 10-µm thin drops to reach a concentration of 40 nM. The cells were collected using TRIzol (Invitrogen). U6 snRNA was purchased from Aidlab, RN3101, China.

**ELISA.** Forty-eight hours after transfection, the old medium was replaced with serum-free medium and cells were incubated for another 24 h. Then the medium was collected to detect the VEGF-A concentration by ELISA assay (Abcam, USA) according to the manufacturer's protocol. Thereafter, the serum-free medium containing different concentrations of VEGF-A (AGS-VEGF-A-medium) was placed on standby at -80˚C for EC proliferation and tube formation assays (24).

**Luciferase reporter assay.** First, we predicted that human miR-125a was able to regulate the expression of VEGF-A, and we aimed to identify the possible site (http://www.microrna.org). The 3'-UTR of human VEGF-A was amplified by PCR (25). The primers for PCR amplification are: forward, 5'-ATCTCAGCATGCTGTCAGTTACCTACTATTGCGGGCCTG-3' and reverse: 5'-GCCCTGAATGCGTACAGGTGATCAAGTGTCATTTGACGTATCGCT-3'. Then the amplified sequence was cloned into the XbaI site of the pG3 control vector (Promega, USA). The mutated putative miR-125a-5p binding site in the 3'-UTR of VEGF-A was generated using the QuickChange Site-directed Mutagenesis kit (Stratagene, Cedar Creek) according to the manufacturer's protocol. The day before transfection, AGS cells were seeded in 24-well plates (5x10^4 cells/well), then we transfected 500 ng VEGF-A-3'-UTR-pG3 and 500 ng miR-125a mimics or control miRNA mimics (GenePharma, China) into cells using Lipofectamine 2000. Forty-eight hours after transfection, luciferase activity was determined using dual-luciferase reporter assay system (Promega) according to the manufacturer's protocol.

**Cell proliferation assay.** Cell proliferation assays were performed using HUVEC essentially as described (26-28). HUVECs were resuspended to a density of 2x10^5/ml. For CCK-8 proliferation assay, 100 µl cell suspension was planted into each well of 96-well plates. The medium was replaced after cell adherence. The AGS-VEGF-A-medium or VEGF-A-medium (VEGF-A recombinant protein, 3 ng/ml, Sino Biological Inc., China) was formulated as conditioned medium containing 2% FBS, and Akt activity inhibitor (MK-2206 2HCI, 10 µM, Selleck, USA) and used to conduct control experiment. The cell proliferation activity was detected using cell counting kit-8 (CCK8) (Beyotime). After cultured for a few days, 10 µl CCK8 was added to each well and incubated for 1 h in the cell incubator. The optical density (OD) value was read at 450 nm wavelength. For plate colony formation assay, 1x10^4 cells were seeded into 6-well plates and cultured for 2 weeks. The colonies were fixed with 4% paraformaldehyde for 10 min and stained with 0.1% crystal violet (Beyotime) for 5 min. Clones with >50 cells were counted (29,30). Experiments were repeated at least 3 times.

**Cell migration assay.** HUVEC migration was evaluated with a transwell system (Corning Costar, USA) that comprised 8-µm inserts in 24-well plates. Cells were co-cultured as described (31,32). Firstly, genetically modified AGS cells or VEGF-A-medium (3 ng/ml) were added into the lower chamber. When AGS cell fusion reached to 100%, the growth medium was replaced with serum-free medium. HUVECs were resuspended in serum-free medium with 0.1% bovine
serum albumin (BSA) to a density of 5x10^5/ml. Then 100 µl medium containing HUVECs were added in each upper chamber (presence or absence of Akt inhibitor). After incubation for 24 h, cells that did not migrate in the upper chamber were removed with a cotton swab. Then the migrated cells were fixed with 4% paraformaldehyde for 20 min. After that, the cells were stained with 0.1% crystal violet (Beyotime) for 5 min and counted and photographed with a fluorescence microscope on x100 magnification. Experiments were repeated at least 3 times.

In vitro tube formation assay. In vitro tube formation assays were performed using HUVEC essentially as described (33,34). Briefly, 50 µl growth factor-reduced Matrigel (BD, USA), which was thawed on ice in advance, were plated in 96-well plates and incubated at 37˚C for 45 min. In order to investigate the effect of AGS-VEGF-A-medium or VEGF-A-medium (absence of FBS, presence or absence of Akt inhibitor) on angiogenesis in HUVECs, the cells (100 µl, 2x10^5 cells/ml) were seeded on the matrigel and incubated at 37˚C with 5% CO_2 for 4 h. After the culture media were removed, the cells were washed twice with PBS. Then stained with 0.1% crystal violet for 5 min and washed with PBS twice again. Images were captured using a digital camera (x100 magnification). Tubes and nodal structures were counted by two independent researchers. Experiments were repeated at least 3 times.

Immunohistochemistry (IHC). The tumor specimens were fixed, dehydrated and embedded in paraffin, then cut into 3-µm thin slices. After dewaxed and rehydrated, they were autoclaved for 2 min and then incubated with 3% hydrogen peroxide for 10 min at room temperature to remove endogenous peroxidase activity. The slices were treated with 5% BSA for 30 min, followed by incubating with Her-2 antibody (ZSGB-BIO, China) and VEGF-A antibody (Santa Cruz) at 4˚C overnight, respectively, then washed in phosphate-buffered saline (PBS) for 2 min and incubate with secondary antibodies (Dako, USA) at 37˚C for 1 h. Then staining was with diaminobenzidine (DAB) substrate chromogen solution for 5 min at room temperature. Her-2 was evaluated by two pathologists independently. Her-2(0), Her-2(1+) and Her-2(2+) were defined as Her-2(-), while Her-2(3+) was defined as Her-2(+). The evaluation of VEGF-A was analyzed using ImageJ software (National Institutes of Health, USA). The raw data was adjusted appropriately to gain normalized score, and the normalized score greater than 2 was designated as VEGF-A positive, while the other negative.
**MVD analysis.** CD31 (Santa Cruz) was detected using IHC staining methods in 73 cases of FFPET to observe MVD in gastric cancer. Evaluation of MVD was performed by two authors independently. MVD in the tumor was determined as described (35,36). In brief, areas rich in tumor tissue and blood vessels were selected at low magnification (x40). Then the blood vessels were counted in the region at high magnification (x100), or even a higher magnification (x200) for some individual vessels. A single independent CD31 staining cell, or microvessel that had formed tubular structure was considered as a countable microvessel. In order to reflect the MVD more accurately, we kept count of five horizons (x100) accumulatively.

**Statistical analysis.** The experimental data were presented as mean ± SD. The differences among variables were assessed by \( \chi^2 \) analysis or 2-tailed Student's t-test. Survival curves were plotted by the Kaplan-Meier method, and differences were assessed by the log-rank test. Correlation parameters were submitted to Pearson's correlation. Statistical analyses were conducted using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). \( P<0.05 \) was considered to have a significant statistical difference.

**Results**

miR-125a regulates the expression of VEGF-A in GC cells. QRT-PCR analysis showed that miR-125a expression in gastric cancer cells (SGC7901, AGS and BGC-823) were lower than that in GES-1 cells or normal gastric tissue (NGT) (Fig. 1A). We transfected miR-125a inhibitors or miR-125a mimics into AGS cells (Fig. 1B and C). Our investigations revealed that the VEGF-A mRNA levels in groups transfected with miR-125a mimics were significantly lower than the control group; in contrast, the VEGF-A mRNA levels were significantly higher in groups transfected with miR-125a inhibitors (Fig. 1D). On the other hand, VEGF-A protein levels detected by western blotting were also reduced in groups that transfected with miR-125a mimics and increased in groups transfected with miR-125a inhibitors (Fig. 1E). Moreover, we obtained a similar result when compared VEGF-A protein levels in the medium of the transfected groups with that in control groups using ELISA technique (Fig. 1F).

**Figure 2.** VEGF-A is a target gene of miR-125a. (A) Binding sites of miR-125a in VEGF-A was predicted. (B) The luciferase reporter assay revealed that miR-125a can result in a significant reduction of the relative luciferase activity while mutating the binding sites abolished this effect. *P<0.05 vs. control group.

VEGF-A is a target gene of miR-125a. Via predictive analysis, we found that miR-125a had binding sites in the 3'-UTR region of VEGF-A mRNA (Fig. 2A). To verify whether miR-125a was capable of regulating the expression of VEGF-A, we performed a luciferase reporter assay. The luciferase reporter assay revealed that miR-125a can result in a significant reduction of the relative luciferase activity while mutating the binding sites abolished this effect (Fig. 2B).

Enhanced migration potential of HUVECs due to increased secretion of VEGF-A in gastric cancer cells. Co-culture transwell assay was used to detect the migration ability of HUVECs. The migration ability of HUVECs declined when HUVECs were co-cultured with miR-125a mimic transfected AGS cells, while the capacity was enhanced in miR-125a inhibitor transfection system. However, this effect can be repealed by Akt inhibitor MK-2206. Moreover, VEGF-A recombinant protein may contribute to the migration of HUVECs. Similarly, this effect can be abolished by MK-2206 (Fig. 3).
Increased secretion of VEGF-A in gastric cancer cells may enhance the proliferation potential of HUVECs. In vitro tube formation assay was performed to detect the angiogenesis potential of HUVECs in different conditioned media. Comparing conditioned medium of control AGS cells with that of miR-125a mimic-transfected AGS cells, the latter contained lower VEGF-A and decreased tube formation (Fig. 4A and B) and nodal structure (Fig. 4A and C); however, conditioned medium of miR-125a inhibitor transfected AGS cells improved the ability of HUVECs to form tube and nodal structures and this effect can be inhibited by Akt inhibitor MK-2206 (Fig. 4A and B). Moreover, exogenous recombinant VEGF-A protein enhanced the tube and nodal structure forming ability of HUVECs, similarly to that shown by MK-2206 (Fig. 4A and C).

Increased secretion of VEGF-A in gastric cancer cells may enhance the angiogenesis potential of HUVECs. The proliferation potential of endothelial cells directly affected angiogenesis; therefore, it was also necessary to detect changes in the proliferation ability of endothelial cells in conditioned medium. We cultured HUVECs with AGS-VEGF-A-medium or VEGF-A-medium containing different concentrations of VEGF-A. The colony formation assay and CCK-8 proliferation assay revealed significant changes in the proliferation capacity of HUVECs. Increased VEGF-A in medium can promote the proliferation of HUVECs, similarly, this effect can be abolished by the Akt inhibitor (Fig. 5A and B). The change in biological function of HUVECs may be associated with the VEGF-A concentration in ambient environment. Increased VEGF-A may contribute to the migration, proliferation and angiogenesis through activation of Akt signaling pathway in endothelial cells, while reduced VEGF-A or inhibition of Akt activity decreased the ability (Fig. 5C).

Relationship between the expression of miR-125a, VEGF-A and MVD in gastric cancer tissues. QRT-PCR was performed to detect the relative expression of miR-125a in GCT and NGT. We found that the expression levels of miR-125a in GCT were lower than that in NGT, which showed a significant statistical difference (p<0.05) (Fig. 6A). The relative expression of miR-125a in tumors scored <1.00 were classified as low expression, while the other cases as high expression. According to this artificial demarcation, the low expression ratio of miR-125a in these 73 cases of gastric cancer tissues was 50.7%. Furthermore, we analyzed the expression of Her-2 and VEGF-A in 73 cases of gastric cancer tissues by IHC. Her-2 was expressed on the membrane of the tumor cells, with a positive rate of 17.8% (Fig. 6B and C). VEGF-A cytoplasmic expression showed positive rate of 31.5% (Fig. 6D and E). In order to reveal the relationship between the expression of miR-125a, VEGF-A and the numbers of tumor microvessels in gastric cancer, we performed IHC staining of vascular marker CD31 in 73 cases of gastric cancer tissues. Single independent endothelial cells (red arrow) or microvessels that had formed a complete tubular structure (black arrow) was considered as a countable microvessel (Fig. 6F and G). Correlation analysis suggested that the expression of miR-125a was inversely proportional to MVD (r=-0.5382) (Fig. 6H), while VEGF-A expression was positively related to MVD (r=0.7226) (Fig. 6I). Moreover, the expression of miR-125a and VEGF-A also showed an inverse correlation (r=-0.4554) (Fig. 6J).

Correlation analysis between miR-125a and clinicopathologic parameters in gastric cancer. We analyzed the correlation between miR-125a and clinicopathological parameters in gastric cancer (Table I). The expression of miR-125a was correlated with lymph node status, the MVD of GC tissues, the clinical stage of GC and the expression of VEGF-A and Her-2 (p<0.05). The clinical significance of miR-125a and VEGF-A in GC was further analyzed based on follow-up of the patients. The low expression of miR-125a indicated a worse prognosis in patients compared to the high expression of miR-125a (Fig. 7A), while low expression of VEGF-A indicated a better prognosis compared to the high expression of VEGF-A (Fig. 7B). MVD was divided into low-density group
Figure 4. VEGF-A promotes the angiogenesis potential of HUVECs. (A) In vitro tube formation assay of the angiogenesis ability of HUVECs in the different groups. (B) Comparison of the ability of HUVECs tube formation in different groups. (C) Comparison of the ability of HUVECs nodal structure formation in different groups. *P<0.05 vs. control group.

Figure 5. VEGF-A promotes the proliferation of HUVECs. (A) CCK-8 proliferation assay of the changes in proliferation capacity of HUVECs after cultured with VEGF-A-medium containing different concentrations of VEGF-A. (B) Colony formation assay of the changes in proliferation capacity of HUVECs after cultured with VEGF-A-medium containing different concentrations of VEGF-A. (C) Effect of medium containing different concentrations of VEGF-A on Akt phosphorylation of HUVECs. *P<0.05 vs. control group.
and high-density group when 150 was set as the critical value. The results indicate that high-density group had a poorer prognosis (Fig. 7C).

**Discussion**

miRNAs have been shown to play important roles in the development of tumors by participating in regulation of gene expression (37–40). miRNAs have been reported as potential new tumor markers in recent years. Therefore, exploring the clinical roles and molecular mechanisms of miRNAs in the malignant development is an effective way to delay the development of GC and improve the prognosis of GC patients. In this study, we mainly investigated the role of miR-125a in angiogenesis in GC.

Substantial evidence has suggested that higher density of tumor blood vessels predict worse prognosis of cancer
In vitro significant difference compared to VEGF-A-negative GC. The number of microvessels in VEGF-A-positive GC had a statistically significant increase compared to VEGF-A-negative GC. In vivo, tumor tissues showed that the growth of GC was attributable to the overexpression of VEGF-A. This study demonstrated that the activation of VEGF-A/VEGFR-2 pathway is sufficient to promote the tumor growth of both primary and metastatic cancer, indicating that VEGF-A plays a key role in tumor angiogenesis (48). This study demonstrated that the growth of GC was attributable to the overexpression of VEGF-A by facilitating angiogenesis, as the expression of VEGF-A was positively related to microvessel quantity in tumor tissues. In vivo, MVD analysis showed that the number of microvessels in VEGF-A-positive GC had a statistically significant difference compared to VEGF-A-negative GC.

miR-125a was an independent prognostic factor in gastric cancer, which may be related to the target gene Her-2 (10,11). Ectopic expression of miR-125a-5p substantially inhibited the proliferation, migration and invasion by targeting E3F3 in gastric cancer cells (51). It has also been reported that miR-125a may inhibit the proliferation and metastasis of hepatocellular carcinoma partly by downregulating MMP11 and VEGF-A (52). However, the relative contribution of miR-125a in regulating VEGF-A in gastric cancer remained unknown. This study demonstrated that miR-125a decreased significantly in gastric cancer. Moreover, the expression of miR-125a was associated with the clinical stage of gastric cancer and the expression of VEGF-A, suggesting that it may become a new biological marker.

miR-125a, VEGF-A, and MVD in tumor tissues can all predict the prognosis in patients. In fact, anti-angiogenesis approach has already been used to treat cancer in recent years. Although it has certain effects, many deficiencies exist (43,53,54). Theoretically, any molecule in miR-125a/VEGF-A/VEGFR2/Akt signaling pathway may become a therapeutic target. However, anti-angiogenic drugs targeting VEGF or its receptors, which aim at blocking the blood supply to tumors, may also cause hypoxia of normal body and thereby fuel tumor progression and treatment resistance. Perhaps targeting the upstream molecules of VEGF-A signaling, such as miR-125a, can achieve a therapeutic effect; however, there is a long way to go yet.

In conclusion, this study provided evidence that miR-125a inhibited the growth of gastric cancer by targeting VEGF-A. Furthermore, increased levels of miR-125a resulted in inhibition of angiogenesis in gastric cancer. Taken together, these findings may help to understand the pathogenesis of gastric cancer in depth and bring new ideas to the treatment of gastric cancer in the future.

Acknowledgements

This study was supported by grants from Natural Science Foundation of China (no. 30570725).


