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Research paper

miR-218 inhibited tumor angiogenesis by targeting ROBO1 in gastric cancer

Zhang Xiangyuan [a,1], Dong Jiaqiang [a,1], He Yan [a,1], Zhao Ming [b], Liu Zhen [a], Wang Na [a], Jiang Mingzuo [a], Zhang Zhe [a], Liu Gang [a], Liu Haiming [c], Nie Yongzhan [a], Fan Daiming [a,*], Tie Jun [a,*]

[a] State Key Laboratory of Cancer Biology, National Clinical Research Center for Digestive Diseases, Xijing Hospital of Digestive Diseases, Fourth Military Medical University, Xi'an, Shaanxi 710032, China
[b] Yan'an University, Yan'an 716000, China
[c] College of Computer Science and Technology, Jilin University, Changchun 120012, China

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ABSTRACT

Aberrant expression of miRNAs is involved in several carcinogenic processes, including tumor growth, metastasis and angiogenesis. The aim of this study was to determine the role of miR-218 in gastric cancer angiogenesis. In situ hybridization was performed on a set of tissue microarray samples to assess the difference in miR-218 expression in vessels between tumor tissues and normal gastric mucosa. In vitro, ectopic expression of miR-218 disturbed the tubular structure and inhibited the migration of endothelial cells. Motility and tube formation were rescued when miR-218 was downregulated. Moreover, miR-218 suppressed endothelial cell sprouting in a fibrin bead sprouting assay. Subsequently, we identified ROBO1 as a target of miR-218 in endothelial cells and determined it was responsible for the effect of miR-218 on tumor angiogenesis. In vivo, local injection of mature miR-218 in xenografted tumors disrupted the vessel plexus and thus inhibited tumor growth. Taken together, our study demonstrated an anti-angiogenic role of miR-218 in gastric cancer and indicated that delivery of miR-218 may be a potential therapeutic strategy to inhibit tumor angiogenesis.

1. Introduction

Angiogenesis, a fundamental hallmark of cancer, is a prerequisite for tumor progression (Hanahan and Weinberg, 2011). Increased angiogenesis not only supplies tumor cells with nutrition but also provides routes for distant metastasis (Chen et al., 2009). Blocking tumor angiogenesis to fight cancer was first proposed in 1971 (Folkman, 1971), and 33 years later, the first anti-angiogenesis drug, bevacizumab, was approved by the FDA (Ellis, 2005). To date, several angiogenesis-inhibiting agents (AIAs) have been used to treat various types of cancers and have shown clinical benefits. However, AIAs in clinical treatment of GC once lagged behind until 2014, a RAINBOW study confirmed the survival advantage of a combined ramucirumab and paclitaxel regimen versus paclitaxel single treatment in patients with previously treated advanced GC (Wilke et al., 2014). Subsequently, ramucirumab was approved by the FDA as the first anti-angiogenic agent targeting VEGFR2 in GC. Despite of the rapid progress, AIA performance is unsatisfactory due to the heterogeneous response among different patients and different cancer types (Ye, 2016). Thus, two strategies, identifying biomarkers to select patients responsive to the treatment or developing novel complementary or alternative therapeutics, such as miRNAs, have been adopted to address this issue. miRNAs have potential advantages in anti-angiogenesis therapy. Most current anti-angiogenesis therapies focus on the VEGF-VEGFR pathway. However, angiogenesis is orchestrated by complex signaling pathways; thus, single target treatment may not be efficient and can be easily by-passed (Weis and Cheresh, 2011). miRNAs regulate multiple signaling pathways through complementary binding to the seed sequence located at the 3′-UTR of target genes, which magnifies their effect. Moreover, miRNAs have been shown to be transmittable between cancer and endothelial cells, remodeling the cancer microenvironment (Zhou et al., 2014; Mao et al., 2015). These characteristics indicate that miRNAs are promising candidates for novel AIAs with a low risk of drug resistance.

miR-218, located in the introns of SLIT2 and SLIT3, directly targets ROBO1, which is in turn a receptor of SLIT2 and SLIT3 (Brose et al.,

Abbreviations: ISH, in situ hybridization; HUVEC, human umbilical vein endothelial cell; HMVEC, human microvascular endothelial cell; EC, endothelial cell; TEC, tumor endothelial cell; AIAs, angiogenesis inhibiting agents; GC, gastric cancer; IHC, immunohistochemistry; MVD, microvessel density; IRS, immunoreactive score; RT, reverse transcription; qPCR, real-time quantitative polymerase chain reaction.

* Corresponding authors at: State Key Laboratory of Cancer Biology, Xijing Hospital of Digestive Diseases, Xijing Hospital, Fourth Military Medical University, China.
E-mail addresses: fandaim@fmmu.edu.cn (F. Daiming), tiejun7776@163.com (T. Jun).
[1] These authors contributed equally to this work.

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1999). We first demonstrated that the SLITs-miR-218-ROBO1 pathway was involved in GC metastasis (Tie et al., 2010; Wang et al., 2015). However, the SLIT-ROBO pathway was originally identified in the central nervous system (Wang et al., 1999). During neural development, the N-terminal fragment of SLIT2 stimulates the formation of axon branches by binding to ROBO1 and ROBO2 (Nguyen Ba-Charvet et al., 2001). Angiogenesis has been shown to share many similarities with axonal guidance, as indicated by the concomitant relationship and molecular cues between nerves and blood vessels (Kerbel and Folkman, 2002). Thus, we hypothesized that miR-218 also contributes to tumor angiogenesis through the SLIT/ROBO pathway. In this study, we systematically examined the role of miR-218 in tumor angiogenesis. Our results showed that miR-218 disrupted the overall structure of vessels in vitro. miR-218-treated xenograft GC tumors showed a reduced blood vessel density and thus a repressed growth rate in vivo. Furthermore, mechanistic analysis demonstrated that the effect of miR-218 was partly mediated by ROBO1. This discovery identified a novel role of miR-218 in GC and indicated it could potentially be used in anti-angiogenic therapy.

2. Material and methods

2.1. Cells and cell culture

Primary human umbilical vein endothelial cells (HUVECs; Sciencell, USA) were cultured in EBM-2 medium supplemented with 5% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% EC growth supplement kit (Sciencell, USA). Only cells in passages 2–8 were used for our experiments. Lung fibroblasts were a kind gift from Dr. Yan of the Fourth Military Medical University, Xi'an, China and were cultured in EGM2 medium (Lonza, USA). Immortalized HUVECs (HUVEC-2C) were purchased from Genechem (China). Immortalized human microvascular endothelial cells (HMVECs) were a generous gift from Dr. Wang of the Fourth Military Medical University. GC cells (BGC-823), HUVECs-2C and HMVECs were all preserved by our laboratory and cultured in DMEM (Gibco, USA) supplemented with 10% FBS (BI, Israel) and 1% penicillin/streptomycin (Gibco, USA). All cells were grown at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Western blot and reverse transcription qPCR (RT-qPCR)

Western blotting was performed using standard procedures. Whole cell lysates were harvested for protein extraction 48 h after transfection. An equal amount (35 μg) of proteins was electrophoresed on 8% polyacrylamide gels and then transferred to nitrocellulose membranes. The membrane was blocked by 5% skim milk, followed by antibody incubation overnight at 4 °C. Antibodies used are listed below: rabbit anti-ROBO1 (ab7279, Abcam) and mouse anti-β-actin (A1978, Sigma). β-Actin was used as the internal reference. RNA extraction was performed using TRIzol (Invitrogen) in both cells and dissected tissues using standard procedures. RT-qPCR was performed on a C1000 touch thermal cycler using a SYBR® Premix Ex Taq™ II kit (TaKaRa, America) according to the instructions from TaKaRa. For miRNAs, stem-loop RT-qPCR was performed, with U6 as the internal reference.

2.3. In vitro cell migration assay and tube formation assay

Two-chamber Transwell assays were performed to assess directional migration as described previously (Tie et al., 2010). For wound healing assays, scratches were made when cells were 80% confluent, and the culture was replaced with fresh basic culture medium after washing 2 times. Pictures were captured every 8 h. The wound healing was analyzed by Image-Pro Plus. For tube formation assays, Matrigel (356234, BD) was placed in 4 °C overnight before use. On the following day, a 96-well plate was coated with 50 μl Matrigel on ice. After 30 min of incubation at 37 °C, HUVECs were added to the plate. Pictures were taken within 24 h. Then, the branch point and length were measured by Image-Pro Plus to assess the tube formation ability.

2.4. Sprouting assay

A fibrin bead sprouting assay was conducted as described by Yan (Nakatsu and Hughes, 2008; Yan et al., 2016). Briefly, after trypsinization, HUVECs were incubated with microcarry beads (Sigma, America) at a ratio of 400 cells per bead. The samples were incubated for 4 h with gentle rotation every 20 min. The mixture was then transferred to a 10 cm^2 dish and cultured at 37 °C overnight. On the following day, beads coated with HUVECs were collected and resuspended in 2 mg/ml fibrinogen solution (Sigma), which contained 0.15 U/ml aprotinin (Sigma). Before the beads were added to a 24-well plate, 0.625 U/ml thrombin (Sigma) was added and mixed with a pipette. Then, 20,000 fibroblasts in 1 ml EGM_2 were added to the above beads. The medium was changed every 2 days. Photos were taken, and sprouting length was calculated by Image-Pro Plus.

2.5. Immunohistochemistry (IHC) and in situ hybridization (ISH)

Written informed consent was obtained from all patients before the tissue samples were used. The protocol was approved by the Institutional Review Board of the Fourth Military Medical University. A set of tissue microarrays containing samples from 182 patients who had undergone surgical resection in Xi'jing Hospital between January 2004 and September 2007 were used for IHC and ISH. Additionally, 162 patients with follow-up data were included in further survival analyses. Standard procedures were used for IHC and ISH. The antibodies for IHC are as follows: CD31 (ab28364, Abcam) and Ki-67 (ab8191, Abcam). A nucleic acid probe for miR-218 was synthesized by Exiqon (Denmark). For quantitative analysis of ISH results, the images were scored based on the intensity and percentage of the positive staining. Scoring is described as follows. The extent of staining was graded as 0, negative; 1, weak; 2, moderate; and 3, strong. The percentage of the positive staining was defined as 0, <5%; 1, 5–25%; 2, 26–50%; 3, 51–75%; and 4, >75% of tumor cells. The immunoreactive score (IRS) was determined by multiplying the two values. Thus, it ranged from 0 to 12 (Zhou et al., 2016).

The microvessel density (MVD) in tissues was calculated by CD31 staining at a magnification of 400×. For each sample, five images of representative areas were acquired to determine the average.

2.6. Transfection and infection assay

For cell transfection, Lipofectamine 2000 (Invitrogen, USA) was used according to the manufacturer's instructions. Then, 50 nM miR-218 mimics and 100 nM inhibitors (RiboBio, China) were transfected into HUVECs to manipulate the cellular expression of miR-218. Stable HUVECs-2C overexpressing miR-218 or with miR-218 silencing were established using lentiviral vectors that expressed either pre-miR-218 or anti-sense miR-218. For rescue experiments, HUVECs-2C-miR-218 were infected with lentiviral vectors expressing the coding sequence (CDS) of ROBO1 followed by puromycin screening.

2.7. Animal experiments

All animal experiments were approved by the Institutional Committee for Animal Research. The experiments were performed as described by Hou (Hou et al., 2011). A total of 10 nude mice (6–8 weeks) were subcutaneously transplanted with 5×10^6 BGC-823 cells per mouse. One week later, when the tumor reached approximately 100 mm^3, every two mice that bore tumors of similar volume were incorporated into a group. For in vivo delivery of miR-218, a micrON™ agomir (RiboBio, China) was used due to its stability and efficiency in vivo. Briefly, for each tumor mass, 5 nmol agomir dissolved in 50 μl sterile