Original Paper

Long Non-Coding RNA HOXA-AS2 Regulates Malignant Glioma Behaviors and Vasculogenic Mimicry Formation via the MiR-373/EGFR Axis

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Key Words

Glioma • Vasculogenic mimicry • Long non-coding RNA • HOXA-AS2 • MicroRNA • MiR-373 • EGFR

Abstract

Background/Aims: Vasculogenic mimicry (VM) has been reported to be a novel glioma neovascularization process. Anti-VM therapy provides new insight into glioma clinical management. In this study, we revealed the role of the long non-coding RNA HOXA cluster antisense RNA 2 (HOXA-AS2) in malignant glioma behaviors and VM formation. **Methods:** Quantitative real-time PCR was performed to determine the expression levels of HOXA-AS2 in glioma samples and glioblastoma cell lines. CD34-periodic acid-Schiff dual-staining was performed to assess VM in glioma samples. CCK-8, transwell, and Matrigel tube formation assays were performed to measure the effects of HOXA-AS2 knockdown on cell viability, migration, invasion, and VM tube formation, respectively. RNA immunoprecipitation, dual-luciferase reporter and Western blot assays were performed to explore the molecular mechanisms underlying the functions of HOXA-AS2 in glioblastoma cells. A nude mouse xenograft model was used to investigate the role of HOXA-AS2 in xenograft glioma growth and VM density. Student's t-tests, one-way ANOVAs followed by Bonferroni posthoc tests, and chi-square tests were used for the statistical analyses. **Results:** HOXA-AS2 was upregulated in glioma samples and cell lines and was positively correlated with VM. HOXA-AS2 knockdown attenuated cell viability, migration, invasion, and VM formation in glioma cells and inhibited the expression of vascular endothelial-cadherin (VE-cadherin), as well as the expression and activity of matrix metalloproteinase matrix metalloproteinase (MMP)-2 and MMP-9. miR-373 was downregulated in glioma samples and cell lines and suppressed malignancy in glioblastoma cells. HOXA-AS2 bound to miR-373 and negatively regulated its expression.

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Epidermal growth factor receptor (EGFR), a target of miR-373, increased the expression levels of VE-cadherin, as well as the expression and activity levels of MMP-2 and MMP-9, via activating phosphatidylinositol 3-kinase/serine/threonine kinase pathways. HOXA-AS2 knockdown combined with miR-373 overexpression yielded optimal tumor suppressive effects and the lowest VM density *in vivo*. **Conclusion:** HOXA-AS2 knockdown inhibited malignant glioma behaviors and VM formation via the miR-373/EGFR axis.

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Introduction

Malignant gliomas are a considerable threat to human health. Despite the improvement in therapeutic approaches, patients with glioblastoma have disappointing median survival times [1]. Angiogenesis contributes significantly to glioma growth, metastasis, and recurrence. However, antiangiogenic therapy, which targets tumor endothelial cells, has limited effects [2]. Vasculogenic mimicry (VM) is described as a novel mechanism of blood supply in gliomas, and endothelium-independent tubular structures are the hallmark of VM [3, 4]. The presence of VM contributes to more aggressive tumor biology and enhances tumor-related mortality [5, 6]. Previous studies have shown that VM may explain the tumor survival mechanism during antiangiogenic therapy [7, 8]. Targeting glioma VM has produced treatment effects in animal models [9]. However, more evidence is needed to explain the mechanisms of glioma VM and the effectiveness of anti-VM therapy.

Non-coding RNAs have been demonstrated to be the predominant transcripts of human DNA. Moreover, recent evidence has implicated the involvement of long non-coding RNAs (lncRNAs) in several of the steps leading to glioma development [10, 11]. The lncRNA HOXA cluster antisense RNA 2 (HOXA-AS2) was first reported to be an apoptosis suppressor in NB4 promyelocytic leukemia cells treated with all-trans retinoic acid [12]. Subsequent studies showed that HOXA-AS2 is aberrantly expressed in several human cancers and functions as an oncogene [13, 14]. However, little is known concerning its expression in glioma and its potential role in glioma malignancy and VM. Multiple mechanisms are involved in lncRNA-mediated tumor biology, among which interactions between lncRNAs and microRNAs are well-studied. By using bioinformatics tools (Starbase 2.0, <http://starbase.sysu.edu.cn/>), we screened a miRNA candidate, miR-373, which may take part in HOXA-AS2-mediated malignant glioma behaviors and VM formation.

EGFR is a crucial receptor-tyrosine kinase and an active target in glioma treatment [15, 16]. Several studies have shown that EGFR is involved in tumor VM formation. By searching online tools (TargetScan 7.1, http://www.targetscan.org/vert_71/; Miranda, <http://www.microrna.org/microrna/home.do>), we found that EGFR is a potential target of miR-373, which indicates that miR-373 may regulate the expression and function of EGFR.

In this study, we hypothesized that lncRNA HOXA-AS2 contributes to malignant glioma behaviors and VM formation by acting as a miR-373 sponge. miR-373 targets EGFR and thus decreases the expression levels of vascular endothelial-cadherin (VE-cadherin), as well as suppresses the expression and activity levels of matrix metalloproteinase (MMP)-2 and MMP-9, via inhibiting the PI3K/AKT pathway.

Materials and Methods

Tissue samples

Normal brain tissues (NBTs) and glioma specimens were collected from the Department of Neurosurgery, Shengjing Hospital of China Medical University. NBTs (5 cases) were obtained from surgeries of brain trauma patients. Glioma specimens were divided into two groups, low grade gliomas (LGGs, WHO I-II, 7 cases), high grade gliomas (HGG, WHO III-IV, 10 cases) according to the diagnosis by neuropathologists. After surgical resection, the tissue samples were collected and stored in liquid nitrogen until use. This study was approved by the Ethics Committee of Shengjing Hospital of China Medical University.

Cell lines and cell culture

Human glioma cell lines (U87 and U251) and human embryonic kidney cell line HEK-293T were purchased from Shanghai Institutes for Biological Sciences Cell Resource Center. Normal human astrocytes (NHAs) were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). U87 and HEK-293 T cells were cultured in glucose Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Carlsbad, CA). U251 cells were cultured in Dulbecco's modified eagle medium/F12 mixed medium supplemented with 10% FBS. NHAs were cultured in astrocyte medium (Carlsbad, CA, USA). All cells were cultured at 37°C in a humidified incubator containing 5% CO₂.

Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was isolated using the Trizol reagent (Life Technologies Corporation). For quantification of HOXA-AS2 and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA, One-Step SYBR PrimeScript RT-PCR Kit (Perfect Real Time; Takara Bio, Inc., Japan) were used according to the manufacturer's instructions. TaqMan MicroRNA Reverse Transcription kit and Taqman Universal Master Mix II (Applied Biosystems, Foster City, CA, USA) were used to detect miR-373 and U6. GAPDH and U6 were used as endogenous controls, respectively. Fold change was calculated as $2^{-\Delta\Delta Ct}$ in gene expression. Primers sequence: HOXA-AS2, forward: TGAACCAGGAATTGTCTCCA, reverse: CATCTCCCACTCCAGAAAG; GAPDH: forward: GGTGAAGGTCGGAGTCAACG, reverse: CCATGTAGTTGAGGTCAATGAAG. Probe: miR-373: 478073 (Applied biosystems); U6: 001973 (Applied biosystems).

CD34- periodic acid-schiff (PAS) dual-straining

For CD34 (1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-74499) straining, standard immunohistochemical staining was performed according to the manufacture's instruction (Maixin Biotechnology Co. Ltd, Fuzhou, China). After strained with 3, 3'-diaminobenzidine, sections were further stained with PAS followed by counterstaining with hematoxylin. Sections were imaged under a light microscope (Olympus, Japan). The VM density was evaluated by three independent observers without knowledge of the outcome.

Cell transfection

The short hairpin RNA against human *HOXA-AS2* (Genebank: NR_122069.1) gene or human *EGFR* (Genebank: NM_005228.4) gene was reconstructed in a pGPU6/Neo vector, respectively (sh-HOXA-AS2, sh-EGFR; GenePharma, Shanghai, China), and its empty vector was used as a negative control (NC) (sh-NC). Human EGFR gene coding sequence was ligated into pIRES2-EGFP vector, (GeneScript, Piscataway, NJ, USA), and its empty vector was used as a NC (EGFR-NC). Glioma cells were seeded in 24-well plates and transfected using Lipofectamine 3000 and P3000 reagents (Invitrogen, CA, USA) when the confluence reached at ~70%. Stable transfected cell lines were selected by the medium supplemented with Geneticin (G418; Sigma-Aldrich, St Louis, MO, USA), G418-resistant glioma cells were obtained after 4 weeks. The transfection of MiRNAs was performed by using agomir-373, antagomir-373, and their respective NC (GenePharma, Shanghai, China), according to the manufacture's instruction. 48 hours after transfection, cells were collected for the subsequent assay. Sequences of shRNA: sh-HOXA-AS2:GCCGTCAGAATCCAAGTGAAT, sh-EGFR:GCGTCCGCAAGTGTAAGAAGT,

Negative Control: GTTCTCCGAACGTGTACAGT.

Cell proliferation assay

Cell proliferation assay was performed by using Cell Count Kit-8 (CCK-8, Beyotime Institute of Biotechnology, Jiangsu, China). Briefly, glioma cells were digested and seeded in 96-well plates at the density of 2000/well. After cultured for 48 h, 10 μ L of CCK-8 were added into each well and incubated at 37°C for 2 h. The absorbance was measured at 450 nm.

Cell migration and invasion assays

The transwell chamber assay was performed to examine the migration and invasion inability of glioma cells *in vitro*. Cells were added in the upper chamber of a 24-well transwell unit (2×10^4 cells/well) with 8 μ m pore size polycarbonate membrane (Corning Costar, Cambridge, MA, USA). The upper chamber contained serum-free medium, while the lower compartment contained medium with 10% FBS; After incubated for

24 h in a humidified atmosphere of 5% CO₂ at 37°C, the cells adhering to the lower surface of the filter were fixed and stained with Giemsa. For the invasion assay, the upper surface of the filter was precoated with 500 ng/μl matrigel solution (BD Biosciences, San Jose, CA, USA). The cells were treated in the same way as the migration assay. Strained cells from at least five random fields were counted.

In vitro tube formation assay

Glioma cells tube formation assay was performed as described previously [4]. Briefly, 24-well plates were coated with Matrigel (100 μl/well, BD Bioscience) and then incubated at 37 °C for 30 min. Then, glioma cells were resuspended in 100 μl complete DMEM at a density of 2×10⁵ cells per ml and added to Matrigel-coated wells. After maintained in 37 °C for 24 h, photos were collected using Olympus DP71 immunofluorescence microscopy (Olympus, Tokyo, Japan). The number of tubular structures in five random fields was quantified using Chemi Imager 5500 V2.03 software.

Western blot analysis

After cell transfection or treatment with PI3K inhibitor (LY294002, Beyotime Institute of Biotechnology), cells were collected, and proteins were isolated using RIPA buffer (Beyotime Institute of Biotechnology). After electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Shanghai, China), the proteins were incubated with primary antibodies (VE-cadherin, 1:1000, Abcam, Cambridge, UK, ab33168, MMP2, 1:1000, proteintech, Wuhan, China, 10373-2-AP; MMP9, 1:1000, proteintech, 10375-2-AP; EGFR, 1:1000, proteintech, 18986-1-AP; PI3K, 1:1000, proteintech, 20584-1-AP; p-PI3K, 1:1000, Cell Signaling Technology, Beverly, MA, USA, #4228, AKT, 1:1000, proteintech, 10176-2-AP; p-AKT, 1:1000, Cell Signaling Technology, #9271; GAPDH, 1:200, Santa Cruz Biotechnology, sc-32233) overnight at 4°C. The membranes were then incubated with respective secondary antibodies for 2 h at room temperature. Bands were visualized using enhanced chemiluminescence (Beyotime Institute of Biotechnology).

Gelatin zymography assay

Zymogram gels were loaded with equal protein amounts. Samples were electrophoretically separated on 8 % sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) containing gelatin (1 mg/ml, Invitrogen, Carlsbad, CA, USA) under nonreducing condition. After washed twice in 2.5 % Triton X-100, the gels were then incubated for 24h with 50 mmol/l Tris-HCl buffer (pH 7.5; containing 0.2 mol/l NaCl and 10 mmol/l CaCl₂). After incubation, Gels were stained with Brilliant Blue R250 and destained. MMP-2 and MMP-9 gelatinolytic bands were visualized on a dark blue background. Band densitometry was determined using ChemImager 5500 V2.03 software.

Luciferase reporter assays

The fragment from HOXA-AS2 containing the predicted miR-373 binding site and its mutant sequence were amplified by PCR, synthesized and cloned into the pmirGLO dual-luciferase vector (Promega, Madison, WI, USA). Similarly, the fragment from EGFR 3'-untranslated regions containing the predicted miR-373 binding site and its mutant sequence were also clone into the above vector. The vectors and agomir-373 or agomir-373-NC were co-transfected into HEK293T cells. Luciferase activity was measured 48 h after transfection through the Dual-Luciferase Reporter System (Promega).

Subcutaneous and orthotopic xenografts in nude mice

All the animal experiments were conducted in accordance with the Animal Welfare Act and were reviewed and approved by the Ethics Committee of Shengjing Hospital. 4–6 weeks old male BALB/c athymic nude mice were purchased from Cancer Institute of the Chinese Academy of Medical Science. Mice were randomized in each double-blind group by three performers, n=7 per group.

To establish subcutaneous xenografts, 5×10⁵ glioma cells were inoculated in the right flank of each mouse. Tumor volumes [(length × width²)/2] were measured by caliper every 4 day until 45 days post-inoculation. To establish orthotopic xenografts, 1×10⁶ cells were injected into the right striatum of the nude mice. The number of survived mice was recorded daily until 45 days when most mice sacrificed. Survival analysis was performed using Kaplan Meier survival curve. Orthotopic xenografts were collected for the CD34-PAS dual-staining.

Statistical analysis

Statistical analysis was performed using GraphPad Prism v5.01. Multiple group comparisons were performed by using a one-way analysis of variance (ANOVA) test followed by Dunnett's post-hoc test. Descriptive data are presented as the mean $\pm$ SD from at least three independent experiments. Differences were considered significant at P values of <0.05 .

Results

HOXA-AS2 was upregulated in glioma samples and glioblastoma cell lines and was positively correlated with glioma VM

To clarify the potential role of HOXA-AS2 in glioma, quantitative real-time PCR (qRT-PCR) was first performed to evaluate the expression of HOXA-AS2 in normal brain tissues (NBTs), low-grade gliomas (LGGs) (grade I-II), high-grade gliomas (HGGs) (grade III-IV), normal human astrocytes (NHAs) and U87 and U251 glioblastoma cell lines. As shown in Fig. 1 A, compared with NBTs, HOXA-AS2 was upregulated in the glioma samples. The expression levels of HOXA-AS2 were also significantly higher in U87 and U251 cell lines than those in the NHAs (Fig. 1 B). To verify the correlation between HOXA-AS2 and glioma VM, CD34-periodic acid-Schiff (PAS) dual-staining was performed. As shown in Fig. 1 C, CD34-negative and PAS-positive channels were considered to be VM. In addition, VM was observed in 3 of the 10 cases of high-grade gliomas. Remarkably, HOXA-AS2 expression levels were higher in the VM-positive group than in the VM-negative group. (Fig. 1 D). Collectively, increased HOXA-AS2 levels may predict glioma VM.

HOXA-AS2 knockdown inhibited glioma cell malignant behaviors and VM formation and reduced the expression levels of VE-cadherin, as well as the expression and activity levels of MMP-2 and MMP-9

Stable HOXA-AS2-knockdown U87 and U251 cells were created to identify the roles of HOXA-AS2 in glioma cells. The transfection efficiency of sh-HOXA-AS2 was shown in Fig. 1 E. The viability of U87 and U251 cells was significantly lower in the sh-HOXA-AS2 groups than that in the sh-NC groups (Fig. 1 F). Similar to the effects on cell viability, HOXA-AS2 knockdown inhibited U87 and U251 cell migration and invasion (Fig. 1 G). *In vitro* VM tube formation assays showed that HOXA-AS2 knockdown decreased tube formation in U87 and U251 cells (Fig. 1 H). The above findings indicated that HOXA-AS2 knockdown suppressed glioma malignancy and VM formation.

MMP-2, MMP-9, and VE-cadherin participate in a crucial signalling pathway in glioma progression and VM formation [17]. Thus, the current study focused on these proteins to investigate the underlying mechanism of HOXA-AS2-mediated glioma biology. Western blot results showed that the protein levels of VE-cadherin, MMP-2, and MMP-9 in U87 and U251 cells were lower in the sh-HOXA-AS2 groups than in the sh-NC groups (Fig. 1 I). In addition, gelatin zymography assays showed that the enzymatic activity levels of MMP-2 and MMP-9 were impaired in the sh-HOXA-AS2 groups (Fig. 1 J).

HOXA-AS2 negatively regulated and bound to miR-373

miR-373 expression levels were measured in U87 and U251 cells in which HOXA-AS2 was stably knocked down. As shown in Fig. 2 A, miR-373 was upregulated in U87 and U251 cells in the sh-HOXA-AS2 groups. lncRNAs can sponge miRNAs and regulate their functions. The potential binding site of miR-373 in HOXA-AS2 was predicted by an online tool (Starbase). Subsequently, dual-luciferase reporter assays were performed to confirm the possible binding site. As shown in Fig. 2 B, luciferase activity was lower in the HOXA-AS2-wt+pre-miR-373 group than in the HOXA-AS2-wt+pre-NC group in HEK293T cells. Furthermore, RNA immunoprecipitation (RIP) assays showed that the relative enrichment of HOXA-AS2 and miR-373 was higher in the Ago2 immunoprecipitants than in the IgG immunoprecipitants. Moreover, in U87 and U251 cells, miR-373 knockdown decreased the

Fig. 1. The expression levels of HOXA-AS2 in glioma and its correlation with glioma VM. (A) The expression of HOXA-AS2 in normal brain tissues (NBTs) and glioma tissues. Data were presented as mean $\pm$ SD from three independent experiments (5 cases in NBTs groups, 7 cases in low grade glioma group (LGGs) and 10 cases in high grade glioma (HGGs) group). * $P < 0.05$ and ** $P < 0.01$ v.s. NBTs group, # $P < 0.05$ v.s. LGGs group. (B) The expression levels of HOXA-AS2 in normal human astrocytes (NHA) and glioma cell lines. Data were presented as mean $\pm$ SD from four independent experiments * $P < 0.05$ v.s. NHA group. (C) CD34-negative and periodic acid-schiff-positive channels in HGGs. (D) Comparing expression of HOXA-AS2 between VM-negative tissues and VM-positive tissues. $\Delta\Delta p < 0.01$ v.s. VM (-) group. (E) HOXA-AS2 was knocked down in U87 and U251 cells. HOXA-AS2 expression levels were determined by qRT-PCR. (F) Cell proliferation was measured by CCK-8. (G) The migration and invasion were measured by transwell assays. (H) The VM formation was measured by Matrigel tube formation assay. (I) The protein expression level of VE-cadherin, MMP-2 and MMP-9 was analyzed by Western blot. (J) The enzymatic activity of MMP2 and MMP9 was analyzed by gelatin zymography assay. Data were presented as mean $\pm$ SD from four independent experiments. * $P < 0.05$ v.s. sh-NC groups.

enrichment of both HOXA-AS2 and miR-373 in the Ago2 immunoprecipitants (Fig. 2 C). The above results indicated that HOXA-AS2 negatively regulated miR-373 via binding to it in a sequence-specific manner.

Fig. 2. HOXA-AS2 negatively regulated miR-373 and bound to miR-373. (A) HOXA-AS2 was knocked down in U87 and U251 cells. The expression level of miR-373 was analyzed by quantitative real-time PCR. Data were presented as mean $\pm$ SD from four independent experiments. * $P < 0.05$ v.s. sh-NC groups (B) Overexpression of miR-373 significantly suppressed the luciferase activity of HOXA-AS2-wt but not HOXA-AS2-mut. Data were presented as mean $\pm$ SD from three independent experiments. # $p < 0.05$ v.s. HOXA-AS2-mut+pre-miR-373 group. (C) Relative enrichment of HOXA-AS2 and miR-373 in Ago2 immunoprecipitates and IgG immunoprecipitates were measured by quantitative real-time PCR. Data were presented as mean $\pm$ SD from three independent experiments. & $P < 0.01$ v.s. IgG groups, $\Delta P < 0.05$ v.s. Control groups.

miR-373 was a tumor suppressor and downregulated the expression of VE-cadherin, as well as the expression and activity levels of MMP-2 and MMP-9

The expression levels of miR-373 in glioma samples and glioma cell lines were determined by qRT-PCR. As shown in Fig. 3 A-B, miR-373 was downregulated in the glioma samples and U87 and U251 cell lines. To further explore the functional role of miR-373, agomir-373, antagomir-373, and their respective negative controls were transfected into U87 and U251 cells. The transfection efficiency of miR-373 was shown in Fig. 3 C. Overexpression of miR-373 decreased the proliferation (Fig. 3 D), migration, invasion (Fig. 3 E), and VM formation (Fig. 3 F) in U87 and U251 cells. Opposite effects were observed in the anti-miR-373 group. Subsequent Western blot results showed that miR-373 overexpression inhibited the expression of VE-cadherin, MMP-2, and MMP-9 and that miR-373 knockdown enhanced the expression of these proteins (Fig. 3 G). Moreover, miR-373 overexpression increased the activity levels of MMP-2 and MMP-9 (Fig. 3 H). The above findings indicated that miR-373 exerted a tumor suppressive function via negatively regulating VE-cadherin, MMP-2, and MMP-9.

miR-373 was involved in HOXA-AS2-mediated glioma malignant behaviors and regulating VE-cadherin, MMP-2, and MMP-9

To explore whether miR-373 was involved in the tumor-suppressive effects of HOXA-AS2 knockdown, co-transfection assays were performed. As shown in Fig. 4 A-C, the combination of HOXA-AS2 knockdown and miR-373 overexpression enhanced the suppressive effects on cell viability, migration, invasion, and VM formation in U87 and U251 cells, which were induced by HOXA-AS2 knockdown or miR-373 overexpression alone. In addition, the co-transfection of sh-HOXA-AS2 and anti-miR-373 reversed the reduction in glioma cell malignancy induced by HOXA-AS2 knockdown and the induction of glioma cell malignancy induced by miR-373 knockdown. Similar to the functional assays, the co-transfection of sh-HOXA-AS2 and pre-miR-373 increased the inhibitory effects on the expression of VE-cadherin and the expression and activity levels of MMP-2 and MMP-9, which were induced

Fig. 3. miR-373 was a tumor suppressor and downregulated the expression of VE-cadherin, the expression and activity of MMP-2, and MMP-9. (A) The expression of miR-373 in normal brain tissues (NBTs) and glioma tissues. Data were presented as mean $\pm$ SD from three independent experiments (5 cases in NBTs groups, 7 cases in low grade glioma group (LGGs) and 10 cases in high grade glioma (HGGs) group). * $P < 0.05$ and ** $P < 0.01$ v.s. NBTs group, # $P < 0.05$ v.s. LGGs group. (B) The expression of miR-373 in normal human astrocytes (NHA) and glioma cell lines. Data were presented as mean $\pm$ SD from four independent experiments & $P < 0.05$ v.s. NHA group. miR-373 was overexpressed and downregulated in U87 and U251 cells. (C) miR-373 expression levels were determined by qRT-PCR. (D) Cell proliferation was measured by CCK-8. (E) The migration and invasion were measured by transwell assays. (F) The VM formation was measured by Matrigel tube formation assay. (G) The protein expression level of VE-cadherin, MMP-2 and MMP-9 was analyzed by Western blot. (H) The enzymatic activity of MMP-2 and MMP-9 was analyzed by gelatin zymography assay. Data were presented as mean $\pm$ SD from four independent experiments. $\Delta P < 0.05$ v.s. pre-NC groups. $\nabla P < 0.05$ v.s. anti-NC groups.

Fig. 4. miR-373 was involved in HOXA-AS2-mediated glioma malignant behaviors and regulation of VE-cadherin, MMP-2, and MMP-9. (A) Stable HOXA-AS2 knocked down U87 and U251 cells were transfected with agomir-373 or antagomir-373. Cell proliferation was measured by CCK-8. (B) The migration and invasion were measured by transwell assays. (C) The VM formation was measured by Matrigel tube formation assay. (D) The protein expression level of VE-cadherin, MMP-2 and MMP-9 was analyzed by Western blot. (E) The enzymatic activity of MMP-2 and MMP-9 was analyzed by gelatin zymography assay. Data were presented as mean $\pm$ SD from four independent experiments. * P < 0.05 v.s. sh-NC+pre-NC groups, # P < 0.05 v.s. sh-HOXA-AS2+pre-NC groups, & P < 0.05 v.s. sh-NC+pre-miR-373 groups, ΔP < 0.05 v.s. sh-NC+anti-NC groups, $\blacktriangle P$ < 0.05 v.s. sh-HOXA-AS2+anti-NC groups, $\blacktriangledown P$ < 0.05 v.s. sh-NC+anti-miR-373 groups.

by HOXA-AS2 knockdown or miR-373 overexpression alone. Moreover, the combination of HOXA-AS2 knockdown and miR-373 knockdown reversed the decrease in VE-cadherin expression levels and MMP-2 and MMP-9 expression and activity levels induced by HOXA-

Fig. 5. EGFR was a target of miR-373. (A) Overexpression of miR-373 significantly suppressed the luciferase activity of wt 3'-UTR of EGFR but not mut 3'-UTR of EGFR. Data were presented as mean $\pm$ SD from three independent experiments. * P < 0.05 v.s. EGFR3'-UTR-mut+pre-miR-373 group. (B) miR-373 was overexpressed and downregulated in U87 and U251 cells. The protein expression level of EGFR was analyzed by Western blot. (C) HOXA-AS2 was knocked down in U87 and U251 cells. The protein expression level of EGFR was analyzed by Western blot. (D) Stable HOXA-AS2 knocked down U87 and U251 cells were transfected with agomir-373 or antagomir-373. The protein expression level of EGFR was analyzed by Western blot. Data were presented as mean $\pm$ SD from four independent experiments. # P < 0.05 v.s. pre-NC groups, & P < 0.05 v.s. anti-NC groups, ^ P < 0.05 v.s. sh-NC groups, ▼ P < 0.01 v.s. sh-NC+pre-NC groups, ▼ P < 0.05 v.s. sh-NC+pre-NC groups, ▽ P < 0.05 v.s. sh-HOXA-AS2+pre-NC groups, ^ P < 0.05 v.s. sh-NC+pre-miR-373 groups, * P < 0.05 v.s. sh-NC+anti-NC groups, ♦ P < 0.05 v.s. sh-HOXA-AS2+anti-NC groups, * P < 0.05 v.s. sh-NC+anti-miR-373 groups.

AS2 downregulation, as well as the increase in VE-cadherin expression levels and MMP-2 and MMP-9 expression and activity levels induced by miR-373 downregulation (Fig. 4 D-E). The above data indicated that the tumor-suppressive effects, the inhibition of VE-cadherin expression, and the inhibition of MMP-2 and MMP-9 expression and activity induced by HOXA-AS2 knockdown were mediated by miR-373 in glioma cells.

EGFR was a target of miR-373

It is widely accepted that miRNAs inhibit gene expression via an RNA-induced silencing complex [18]. Online tools show that the 3'-untranslated region (UTRs) of VE-cadherin, MMP-2, and MMP-9 do not have a miR-373 putative binding site, which indicates that miR-373 regulates VE-cadherin, MMP-2, and MMP-9 in an indirect way. By searching online tools, EGFR was screened to be a potential target of miR-373. Subsequently, we performed dual-luciferase reporter assays to confirm the above binding site. As shown in Fig. 5 A, luciferase activity in the HEK293T cells co-transfected with EGFR-wt and pre-miR-373 was lower than in the cells co-transfected with EGFR-wt and pre-NC. Furthermore, Western blot assays showed that miR-373 overexpression inhibited the expression of EGFR in U87 and U251 cells, and opposite effects were observed in the miR-373 knockdown group (Fig. 5 B). In addition, HOXA-AS2 knockdown decreased EGFR expression levels in U87 and U251 cells

Fig. 6. EGFR increased glioma cells VM formation and the expression of VE-cadherin, and the expression and activity of MMP-2 and MMP-9 by activating PI3K/AKT pathway. EGFR was overexpressed and downregulated in U87 and U251 cells. (A) EGFR expression levels were determined by Western blot. (B) The VM formation was measured by Matrigel tube formation assay. Data were presented as mean $\pm$ SD from three independent experiments $\Delta P < 0.05$ v.s. EGFR-NC groups, $\nabla P < 0.05$ v.s. sh-NC groups, $**P < 0.01$ v.s. EGFR-NC groups, $##P < 0.01$ v.s. sh-NC groups. (C) The PI3K/AKT pathway was analyzed by Western blot. Data were presented as mean $\pm$ SD from four independent experiments. $*P < 0.05$ v.s. EGFR-NC groups, $\#P < 0.05$ v.s. sh-NC groups. (D) PI3K inhibitor (LY294002) was introduced in U87 and U251 cells that stably overexpressed or knocked down EGFR. The protein expression level of VE-cadherin, MMP-2 and MMP-9 was analyzed by Western blot. (E) The enzymatic activity of MMP-2 and MMP-9 was analyzed by gelatin zymography assay. Data were presented as mean $\pm$ SD from four independent experiments. $\Delta P < 0.05$ v.s. EGFR-NC groups, $\Delta P < 0.01$ v.s. EGFR groups, $\nabla P < 0.05$ v.s. sh-NC groups, $\Delta P < 0.05$ v.s. sh-EGFR groups.

(Fig. 5 C), and miR-373 was involved in the above effects (Fig. 5 D). These findings revealed that HOXA-AS2 knockdown inhibited EGFR expression levels via upregulating miR-373.

EGFR enhanced glioma VM formation and increased the expression levels of VE-cadherin, as well as the expression and activity levels of MMP-2 and MMP-9, by activating the PI3K/AKT pathway

EGFR promotes malignant behaviors in glioma cells [19]. To determine whether EGFR regulates glioma VM formation, gain- and loss-of-function assays were performed. The

Fig. 7. HOXA-AS2 knockdown combined with miR-373 overexpression suppressed tumor growth and glioma VM formation in vivo. (A) Effect of HOXA-AS2 knockdown, miR-373 overexpression and combination of HOXA-AS2 knockdown and miR-373 overexpression on glioma xenograft volume and nude mice survival. Data represent mean $\pm$ SD. (n=7, each). *P<0.05 v.s. sh-NC+pre-NC groups, **P<0.01 v.s. sh-NC+pre-NC groups, #P<0.05 v.s. sh-HOXA-AS2 groups, &P<0.05 vs pre-miR-373 groups. (B) Effect of HOXA-AS2 knockdown, miR-373 overexpression and combination of HOXA-AS2 knockdown and miR-373 overexpression on glioma xenograft VM density. Data were presented as mean $\pm$ SD from four independent experiments. *P<0.05, **P<0.01, ##P<0.01, &P<0.05.

transfection efficiency of EGFR was shown in Fig. 6 A. As shown in Fig. 6 B, in U87 and U251 cells, VM tube formation was promoted in the EGFR overexpression groups, and a significant decrease in VM tube formation was observed in the sh-EGFR group. Mechanism research showed that EGFR overexpression increased the expression levels of VE-cadherin, as well as the expression and activity levels of MMP-2 and MMP-9, via activating the PI3K/AKT pathway (Fig. 6 C); these effects could be blocked by using a PI3K inhibitor. In addition, the combination of EGFR knockdown and a PI3K inhibitor had the strongest inhibitory effects on VE-cadherin, MMP-2, and MMP-9 (Fig. 6 D-F).

HOXA-AS2 knockdown combined with miR-373 overexpression yielded optimal tumor-suppressive effects and the lowest VM density in vivo

A subcutaneous xenograft study and an orthotopic xenograft study were performed to further confirm the above findings. As shown in Fig. 7 A, there were no significant differences in the average volumes of the xenograft gliomas between the control and sh-NC +pre-NC groups. The average xenograft volume was smaller in the sh-HOXA-AS2 group, pre-miR-373 groups, and sh-HOXA-AS2 + pre-miR-373 groups. Among all the groups, the sh-HOXA-AS2 + pre-miR-373 groups had the smallest tumor volumes. Similar to the results of the subcutaneous xenograft study, the orthotopic xenograft study showed that mice in the sh-HOXA-AS2 + pre-miR-373 group had the longest survival time. Moreover, CD34-PAS dual-staining showed that xenograft gliomas in the sh-HOXA-AS2 + pre-miR-373 group had the lowest VM densities (Fig. 7 B).

Discussion

In the present study, we found that lncRNA HOXA-AS2 was upregulated in glioma samples and glioma cell lines. Moreover, there was a positive correlation between HOXA-AS2

expression levels and glioma VM. HOXA-AS2 knockdown inhibited cell viability, migration, invasion, and VM formation in glioma cells. In contrast, miR-373 was downregulated and exerted a tumor suppressive role in glioma cells. EGFR was found to be a target of miR-373. HOXA-AS2 knockdown decreased EGFR expression levels via binding to and upregulating miR-373. EGFR promoted the expression of VE-cadherin, as well as increased the expression and activity levels of MMP-2 and MMP-9, through activating the PI3K/AKT pathway. Fig. 8 shows the underlying mechanism of the HOXA-AS2/miR-373/EGFR pathway in the regulation of U87 and U251 malignant cell behaviors and VM formation.

Recently, a growing number of publications have shown that lncRNAs are deregulated in malignant tumors and participate in carcinogenesis [20, 21]. In addition, lncRNAs also modulate glioma biology, such as migration, invasion, angiogenesis, and drug resistance [10, 11, 22]. In this study, we demonstrated that lncRNA HOXA-AS2 contributed to cell viability, migration, invasion, and VM formation in glioma cells. Consistent with our findings, HOXA-AS2 was proven to facilitate tumor malignancy in several publications. *In vitro* and *in vivo* studies [23] showed that HOXA-AS2 overexpression promotes cell proliferation in gastric cancer. In addition, Zhang et al. [24] demonstrated that HOXA-AS2 overexpression promotes tumor cell proliferation and induces epithelial-mesenchymal transition (EMT) in gallbladder carcinoma. It has been confirmed that lncRNAs regulate tumor progression through modulating gene expression at the epigenetic, transcriptional and posttranscriptional levels, in which interaction with miRNAs is important. For instance, HOTAIR epigenetically silences miR-34a, leading to enhanced EMT in human gastric cancer [25], and MIR31HG exhibits oncogenic properties by sponging miR-193b in pancreatic ductal adenocarcinoma [26]. Our results showed that HOXA-AS2 negatively regulated miR-373 expression by sponging it. Xie et al. [23] showed that HOXA-AS2 epigenetically silences P21, PLK3, and DDIT3 in gastric cancer cells via binding to EZH2. Moreover, Ding et al. [13] proved that HOXA-AS2 recruits EZH2 and LSD1 to the *p21* and *KLF12* promoter regions, which inhibits *p21* and *KLF12* translation. Accordingly, further investigations of whether other mechanisms are involved in HOXA-AS2-mediated miR-373 expression changes should be performed.

Since the first results in melanoma [27], VM has been identified and proven functional in several types of aggressive tumors, including glioma and glioma xenografts [3, 28-30]. Tumor cells, which line the VM channel, tend to migrate and metastasize to other regions as they are directly exposed to blood flow [31, 32]. VM is an aggressive-tumor-specific phenomenon; thus, treatment targeting VM would have fewer side effects. Liu et al. [5] demonstrated that VM-positive glioma patients have a poorer prognosis than patients with VM-negative gliomas. Ju et al. [9], Huang et al. [33], and Ying et al. [34] proved that liposomes containing VM-targeting drugs improve anti-glioma therapy in orthotopic glioma-bearing nude mice. In agreement with the above findings, our *in vitro* and *in vivo* results showed that the destruction of VM formation by HOXA-AS2 knockdown impaired glioma malignancy.

VE-cadherin, MMP-2, and MMP-9 were proven to contribute considerably to VM formation. Tumor cells that generate VM express genes that used to be considered specific to endothelial cells, epithelial cells, and fibroblasts [35, 36]. Among these genes, VE-cadherin is a key modulator of VM [37]. VE-cadherin deficiency decreases VM tube formation in melanoma [38]. In addition, VE-cadherin mediates the cleavage of the laminin5 γ 2 chain, which may result in VM formation [39, 40]. Recent studies showed

Fig. 8. The Schematic representation of the mechanism underlying the HOXA/AS2-miR-373/EGFR axis in U87 and U251 cells.

that VE-cadherin plays prominent roles in glioma VM formation [41, 42]. Furthermore, MMPs enhance extracellular matrix degradation, which is related to migration, metastasis, and VM formation in tumor cells. In ovarian cancer cells, MMP-2 and MMP-9 have been shown to be upregulated and activated in CD147-induced VM formation [43]. Moreover, MMP-2 and MMP-9 mRNA and protein levels are downregulated in the Rg3-mediated inhibition of pancreatic cancer VM formation [44]. In the present study, we proved that VE-cadherin, MMP-2, and MMP-9 were involved in HOXA-AS2-mediated glioma VM formation. Certainly, the precise mechanism needs to be comprehensively explored.

miR-373 functions as either an oncogene or a tumor suppressor in certain types of cancer. For example, miR-373 acts as an oncogene in cervical cancer [45] and esophageal squamous cell carcinoma [46]. However, miR-373 is downregulated in epithelial ovarian cancer [47] and pancreatic cancer [48] and exerts tumor-suppressive effects. In this study, we found that miR-373 played a tumor suppressor role in human glioma through targeting EGFR. Associations between miRNAs and VM were also confirmed by previous studies. Zhao et al. [49] showed that miR-186 regulates prostate cancer VM formation via targeting Twist1. In addition, Wan et al. [50] demonstrated that miR-124 overexpression suppresses VM formation by inhibiting *amotL1* expression in cervical cancer cells. Moreover, miR-26b [51], miR-9 [52], and miR-let-7f [53] were reported to inhibit glioma VM formation. Consistent with this evidence, the results in this study showed that the ectopic expression of miR-373 reduced VM formation in U87 and U251 glioma cells. Wei et al. [54] showed that miR-373 inhibits U251 glioma cell migration and invasion but does not affect proliferation in U251 cells. However, our results showed that miR-373 suppressed malignant behaviors in U87 and U251 cells, including cell viability. These disparate results may be attributed to the different assay systems.

EGFR is a widely studied receptor-tyrosine kinase in experimental tumor studies and clinical treatment. Recent findings showed that EGFR is involved in tumor VM formation. An *in vitro* study by Lee et al. [55] demonstrated that inhibiting EGFR phosphorylation or EGFR knockdown suppresses VM formation in breast cancer stem/progenitor cells (BCSCs). In addition, Tu et al. [56] confirmed that hinokitiol decreases EGFR protein stability, leading to the destruction of VM in BCSCs. EGFR also regulates glioma VM formation. Zhang et al. [57] proposed that the inhibitory effects of LRIG1 on glioma VM formation are most likely mediated by the suppression of the EGFR/PI3K/AKT pathway. In this research, we demonstrated that EGFR enhanced glioma VM formation via increasing the expression levels of VE-cadherin, as well as the expression and activity levels of MMP-2 and MMP-9. Previous studies have shown that EGFR regulates VE-cadherin, MMP-2, and MMP-9 expression and function via the PI3K/AKT pathway, MEK/ERK pathway, and AKT pathway [58, 59]. The present study showed that EGFR increased VE-cadherin expression levels and MMP-2 and MMP-9 expression and activity levels via activating the PI3K/AKT pathway.

Conclusion

This study found for the first time that HOXA-AS2 was upregulated in glioma samples and cell lines and was positively associated with glioma VM. HOXA-AS2 knockdown remarkably inhibited glioma malignancy and VM formation and the expression of EGFR by upregulating miR-373. These findings may provide new insights into glioma treatment.

Disclosure Statement

The authors declare that they have no competing interests.

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YX and YL conceived and designed the project. YG and WG performed most of the experiments. JZ and JC acquired clinical data and did statistics analysis. YH and XL wrote the manuscript. YT and LZ did animal experiments and Western blotting experiments. YX and YL contributed to the manuscript revision.

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