Involvement of microRNA-1297, a new regulator of HMGA1, in the regulation of glioma cell growth in vivo and in vitro

Jiachong Wang1,2, Xiaoyun Xu2, Shaowei Mo2, Ye Tian1, Jian Wu3, Jianning Zhang1, Jiannong Zhao2

1Tianjin Neurological Institute, Department of Neurosurgery, General Hospital, Tianjin Medical University, Tianjin 300052, China; 2Department of Neurosurgery, The People’s Hospital of Hainan Province, Haikou 570311, Hainan, China; 3Department of Laboratory Medicine, The First People’s Hospital of Yancheng City, Yancheng 224005, Jiangsu, China

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Abstract: MicroRNAs (miRNAs) are a class of versatile gene expression regulators, participating in the regulation of gene expression at the post-transcriptional level in both physiological and pathological conditions. Gliomas are the most common brain malignancy in adults, and deregulation of microRNAs takes part in the gliomagenesis process. Here, we found that the expression of miR-1297 is significantly reduced in both glioma cell lines and clinical glioma tissues. Using the MTT assay, soft agar colony formation assay and xenograft tumor formation assay, we show that miR-1297 is a tumor suppressor microRNA in gliomas. We demonstrate that the high mobility group protein A1 (HMGA1) is the functional target of miR-1297 in glioma cells. HMGA1 significantly promotes the growth of glioma cells both in vitro and in vivo. Together, we unveil a new molecular mechanism in gliomas that may shed new light on understanding this brain malignancy.

Keywords: miR-1297, HMGA1, glioma, cell growth

Introduction

Gliomas, characterized by uncontrolled proliferation, robust angiogenesis, and invasion into the surrounding normal brain tissue, account for approximately 50% of brain tumors among adults [1] and are the most common malignant primary brain tumors [2]. There are several origins of glioma cells, such as astrocytes, oligodendrocytes, and ependymal cells, resulting in the heterogeneity of gliomas [3]. Gliomas are highly progressive malignancies with a poor prognosis [4] and a short median survival time of approximately 14.6 months [5, 6]. An urgent need exists to uncover the molecular basis of gliomas in order to develop new, highly efficient therapeutic methods for this life-threatening disease.

MicroRNAs are a class of small RNA molecules, ~22 nt in length, which participate in gene regulation based upon the sequence of their target genes [7-9]. MicroRNAs regulate the expression of genes that play key roles in both physiological and diseased states [10]. During carcinogenesis, the expression of numerous microRNAs is altered [11, 12], resulting in the activation of oncogenes or the inactivation of tumor suppressor genes. Many microRNAs have been reported to be dysregulated in gliomas [13-15], affecting the cell cycle, apoptosis and migration of glioma cells. MicroRNA-1297 is a tumor suppressor microRNA in many types of human cancers, including colorectal cancer [16], hepatocellular cancer [17, 18] and lung cancer [19]. However, the role of miR-1297 in gliomas is not well understood.

The high mobility group protein A1 (HMGA1) is one member of the HMGA protein family, which participates in the regulation of the chromatin structure by direct interactions with A/T-rich DNA sequences located in the promoter and enhancer regions of a large variety of genes [20]. Previous studies indicate that HMGA1 participates in the process of cancer [21-24] and can serve as a biomarker for cancer diagnosis and therapy [23]. However, its role and the regulation of its expression in gliomas are not well understood.
In the present study, we found that the expression of miR-1297 is significantly reduced in various types of glioma cell lines and clinical glioma specimens. Re-expression of miR-1297 in U87 and U251 glioma cells results in slower proliferation and lower cellular viability both in vitro and in vivo. We performed a series of experiments to demonstrate that HMGA1 is a direct target gene of miR-1297 and is negatively regulated by miR-1297 in glioma cells. HMGA1 can significantly increase the cellular viability and growth of glioma cells both in vitro and in vivo. Downregulation of miR-1297 in glioma cells and clinical glioma tissues result in enhanced expression of HMGA1.

Materials and methods

Cell lines and clinical tissue samples

The glioma cell lines used in this study were maintained in liquid nitrogen in our laboratory and were cultured in medium as recommended by the ATCC. All of the transfections were performed using Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s introductions.

Ten pairs of clinical glioma specimens and their respective adjacent non-cancerous tissues were obtained from The People’s Hospital of Hainan Province after an informed consent was completed by the patients.

Plasmid construction

The expression plasmid for miR-1297 was constructed with the following primer pair: sense primer: 5’-ATTTTCATAGGACAACATCTTCAAC-3’; and anti-sense primer: 5’-GGCTTTAAAAGTCA-AGGAAAGTTGATAAG-3’. The HMGA1 3’UTR reporter plasmid was constructed by inserting HMGA1 3’UTR containing the miR-1297 target site downstream of a firefly luciferase open reading frame; the 3’UTR fragments of HMGA1 were obtained by PCR with the following primer pair: sense primer: 5’-AGGTTTAAAGTTCA-AGGTTTAAAGTTCA-3’ and anti-sense primer: 5’-CAGGGCCTTGGCCCTCACCTTGAGC-3’. Other plasmids used in this study were constructed previously in our laboratory.

RNA isolation and qRT-PCR

Total RNAs were extracted with Trizol reagent (Sigma-Aldrich, USA). The first cDNA strand was obtained by reverse transcription using a random primer, miR-1297 primer or U6-specific RT primer. Real-time PCR was performed using IQ SYBR Green Supermix and the iCycler real-time PCR detection system (Bio-Rad). The change in expression of each target RNA relative to U6 snRNA or beta-actin mRNA was calculated based on the threshold cycle (Ct) as $2^{-\Delta\Delta Ct}$, where $\Delta Ct = Ct_{target} - Ct_{U6/actin}$, and $\Delta(\Delta Ct) = \Delta Ct_{sample} - \Delta Ct_{control}$.

Flow cytometry

The cells were transfected with the indicated plasmids or oligonucleotides for 24 hours before harvesting. The cells were washed with ice-cold 1 x PBS twice, resuspended in FITC-conjugated annexin v binding buffer, and mixed with 5 µl of FITC-conjugated annexin v (Invitrogen, Carlsbad, CA). After 15 min incubation at room temperature in the dark, 5 µl of PI was added, incubated for 10 min, and the cells were analyzed by flow cytometry.

Soft agar colony formation assay

Each well of a 24-well culture dish was coated with 0.5 ml bottom agar-medium mixture (DMEM, 10% FBS, 0.6% agar). After solidifying, 1 ml top agar-medium mixture (DMEM, 10% FBS, 0.3% agar) containing approximately 400 cells was added, followed by another 0.5 ml complete culture medium added to top of the well, and the dishes were incubated at 37°C for 15 days. Colonies were fixed with 6.0% glutaraldehyde, stained with 0.5% crystal violet, and counted using a dissection microscope to determine the plating efficiency.

Xenograft tumor formation assay

U87 glioma cells were transfected with pri-miR-1297 or si-HMGA1 or their respective control plasmid or oligos for 24 hours before harvesting. Approximately $5 \times 10^6$ cells suspended in 100 µl serum-free DMEM culture medium were injected subcutaneously into the flank of nude mice. The tumor sizes were measured every day beginning at day 7 post injection. The tumor volume was calculated as follows: length $\times$ width$^2 \times 1/2$. All mice were sacrificed at day 35 post-injection. The tumors were harvested from mice and stored at -80°C for analysis of miR-1297 or HMGA1 protein. All experiments adhered to national and international standards.
MicroRNA-1297 targets HMGA1 in glioma

Results

miR-1297 is significantly reduced in glioma cells and in clinical glioma specimens

Previous studies had demonstrated that miR-1297 functions as a tumor suppressor microRNA in hepatocellular carcinoma [17, 18] and colorectal cancer [16], but its role in glioma is still unknown. We first measured the expression of miR-1297 in normal human astrocytes and U87, U251, A172, LN340, TJ905 and U373 glioma cell lines. As shown in Figure 1A, in contrast to normal human astrocytes, the expression of miR-1297 in glioma cells was significantly reduced, indicating that in glioma cells, miR-1297 may function as a tumor suppressor microRNA. To confirm the reduced expression of miR-1297 in glioma cells, we measured the expression of miR-1297 in clinical glioma specimens. A significant reduction of miR-1297 in glioma tissues compared with that of adjacent non-cancerous tissues was observed (Figure 1B). Together, these results demonstrated that the expression plasmid of miR-1297 could significantly increase the expression of miR-1297 in U87 and U251 glioma cells (Figure 2A). Exogenous expression of miR-1297 significantly reduced the cellular viability of both U87 and U251 cells as determined using the MTT assay (Figure 2B), indicating a tumor suppressor function for miR-1297 in glioma cells. To further investigate the possible function of miR-1297 in glioma cells, we conducted a soft agar colony formation assay to evaluate the impact of miR-1297 on the growth of glioma cells. As shown in Figure 2C, miR-1297 significantly reduced the colony formation capacity of glioma cells. The regulation of apoptosis is one of the major mechanisms through which microRNAs reduce the viability of cancer cells. Based upon the MTT assay results, miR-1297 significantly increased the apoptosis rate of both U87 and U251 glioma cells (Figure 2D and 2E). Accelerated cell cycle progression is responsible for the high growth rate of cancers. We found that miR-1297 reduced the colony formation capacity of glioma cells, but how did miR-1297 achieve this? To address this question, we analyzed the cell cycle of glioma cells transfected with the miR-1297 expression plasmid. miR-1297 induced a cell cycle arrest in G1/S phase (Figure 2F and 2G). Overall, these results indicate that miR-1297 inhibited the growth of glioma cells by inducing a G1/S cell cycle arrest and reduced the viability of glioma cells by increasing the apoptosis of glioma cells, dem-

Figure 1. The expression of miR-1297 in glioma cell lines and clinical glioma tissues. A. The expression of miR-1297 in various glioma cell lines and normal human astrocytes (NHA) was detected by qRT-PCR; the expression of miR-1297 was normalized to U6 snRNA, and the relative expression of miR-1297 was set to one in the NHA group. B. The expression level of miR-1297 in clinical glioma tissues and adjacent non-cancerous tissues was determined by qRT-PCR. The diagram indicated the ratio of miR-1297 in cancer tissues relative to that of non-cancerous tissues. *p≤0.05.

miR-1297 promotes the apoptosis of glioma cells and inhibits the growth of glioma cells

To investigate the role of miR-1297 in glioma cells, we first constructed miR-1297 expression plasmid. The result of qRT-PCR demonstrated
Figure 2. The impact of miR-1297 on glioma cells. (A) The efficacy of miR-1297 overexpression plasmid in U87 and U251 glioma cells was confirmed by qRT-PCR; the expression of miR-1297 was normalized to U6 snRNA, and the relative expression of miR-1297 was set to one for the pcDNA3-treated group. (B) The effect of miR-1297 on the viability of U87 and U251 glioma cells was determined by MTT assay. (C) The effect of miR-1297 on the colony formation capacity of U87 and U251 glioma cells was measured by colony formation assay. (D and E) The effect of miR-1297 on the apoptosis of U87 and U251 cells was measured by flow cytometry; (D) Shows the quantification of (E). (F and G) The effect of miR-1297 on the cell cycle of U87 and U251 cells was analyzed by flow cytometry. *p<0.05.
MicroRNA-1297 targets HMGA1 in glioma

MicroRNA-1297 demonstrates a tumor suppressor role for miR-1297 in glioma cells.

HMGA1 is a direct target of miR-1297 and is negatively regulated by miR-1297

MicroRNAs elect their function mainly via regulating the expression of their target genes. To identify the target genes of miR-1297 responsible for its biological function in glioma cells, we first predicted the target gene of miR-1297 using the online algorithm Targetscan. We found that HMGA1 is a predicted target of miR-1297 (Figure 3A). To confirm the regulation of HMGA1 by miR-1297, we constructed a luciferase reporter plasmid containing a miR-1297 target site within the HMGA1 3'UTR. miR-1297 significantly reduced the expression of the luciferase reporter in glioma cells (Figure 3B). The regulation of miR-1297 based upon the luciferase reporter was dependent on its target site within the HMGA1 3'UTR (Figure 3B). We further investigated the effect of miR-1297 on endogenous HMGA1 by western blot. miR-1297 reduced the protein level of HMGA1 in glioma cells (Figure 3C). To further confirm the regulation of HMGA1 by miR-1297, we measured the HMGA1 level in various glioma cell lines and normal human astrocytes. Compared with normal human astrocytes, HMGA1 mRNA was significantly increased in glioma cells (Figure 3D), consistent with the reduction of miR-1297 in glioma cells. The results of clinical glioma specimens further sup-
Figure 4. The effect of HMGA1 on U87 and U251 cells. (A and B) The efficacy of si-HMGA1 in U251 and U87 glioma cells was determined by western blot, and GAPDH served as a loading control. (C) The effect of HMGA1 on the viability of U87 and U251 cells was measured by MTT assay. (D and E) The effect of HMGA1 on the apoptosis of U87 and U251 cells was measured by flow cytometry; (D) Shows the quantification of (E). *p≤0.05.
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Figure 5. miR-1297 inhibits the growth of xenograft tumors in vivo. (A) Approximately $5 \times 10^5$ U87 glioma cells with indicated treatments were injected subcutaneously into the flanks of nude mice, and the growth curve of the xenograft tumor was plotted. (B) The mice were sacrificed at day 35 post injection; shown was the tumors forming by indicating treated cells. (C) The mean weight of the tumors in each group in (A and B) was shown. (D) The expression level of miR-1297 in the xenograft tumors formed by the indicated cells was measured by qRT-PCR, with U6 snRNA as an inner control. (E) The expression level of HMGA1 protein level in the xenograft tumors formed by the indicated cells was measured by western blot, and GAPDH served as a loading control. *p≤0.05.

ported the notion of a negative regulation of HMGA1 by miR-1297 (Figure 3E). Together, the above results demonstrate that miR-1297 suppresses the expression of HMGA1 in glioma cells.

**HMGA1 increases the cellular viability of glioma cells**

We demonstrated that miR-1297 could negatively regulate the expression of HMGA1 in glioma cells and function as a tumor suppressor microRNA. What is the role of HMGA1 in glioma cells? Transfected U87 and U251 glioma cells with si-HMGA1 could significantly reduce the protein level of HMGA1 (Figure 4A and 4B). Additionally, si-HMGA1 could significantly reduce the viability of glioma cells (Figure 4C), indicating that HMGA1 may function as an oncogene in glioma cells. We next investigated the role of HMGA1 on the apoptosis of glioma cells. The results demonstrated that HMGA1 could inhibit the apoptosis of glioma cells (Figure 4D and 4E). Overall, these results demonstrate that HMGA1 inhibits the apoptosis, thereby increasing the cellular viability of glioma cells.

**miR-1297 inhibits the growth of xenograft tumor in vivo**

To further validate the tumor suppressor role of miR-1297 in glioma cells, we conducted a xenograft tumor formation assay using cells transfected with the miR-1297 expression plasmid. Compared with the control group, miR-1297 significantly inhibited the growth of the xenograft tumor (Figure 5A-C), consistent with its tumor suppressor role in glioma. To validate that both miR-1297 and HMGA1 participate in the regulation of the growth of the xenograft tumor, we measured the expression of miR-1297 and HMGA1 protein in the xenograft tumors. Indeed, the expression of miR-1297 was significantly increased in the xenograft tumor formed by miR-1297-treated cells compared to the control.
MicroRNA-1297 targets HMGA1 in glioma

pared with the control group (Figure 5D). The protein level of HMGA1 was also significantly reduced in the xenograft tumor in the miR-1297-treated group (Figure 5E). Together, these results indicate that miR-1297 inhibits the growth of glioma in vivo by suppressing the expression of HMGA1, further supporting its tumor suppressor role in glioma.

**HMGA1 promotes the growth of xenograft tumor in vivo**

To further confirm the tumor-promoting function of HMGA1 in glioma, we performed a xenograft tumor formation assay using U87 cells transfected with si-NC or si-HMGA1. Compared with si-NC treated group, the growth of xenograft tumor formed by si-HMGA1-treated cells was slow (Figure 6A). The mean weight of tumors in the si-HMGA1-treated group was significantly reduced (Figure 6B). These results support the oncogene-like function of HMGA1 in glioma.

Overall, the results presented here demonstrate that miR-1297 participates in gliomagenesis by inhibiting the expression of HMGA1 and that its downregulation in glioma cells may promote the progression of glioma.

**Discussion**

Resistance to apoptosis and uncontrolled cell growth and proliferation are the two most common characteristics of malignant tumors, which render cancers difficult to treat. Gliomas are the most common brain malignancy. Despite treatments, including radiotherapy, surgery, and chemotherapy, the quality of life and extended survival rate remain poor. An urgent need exists to clarify the molecular mechanisms of gliomas and help develop new therapeutic methods.

Previous studies have demonstrated that microRNAs participate in gliomagenesis. miR-132 inhibits the growth and migration of glioma cells by regulating the expression of MMP16 [13]. miR-218-5p regulates the stem cell properties of glioma cells [14]. In the present study, we sought to define the role of miR-1297 and its target gene in glioma cells. The biological function of miR-1297 is not well understood, particularly its role in gliomas. Previously, miR-1297 has been demonstrated to be an oncomiR in laryngeal squamous cell carcinoma (LSCC) [25] and testicular germ cell tumors (TGCT) [26]. However, its tumor-promoting role is controversial because in colorectal cancer [16], hepatocellular cancer [18] and lung adenocarcinoma [19], miR-1297 functions as a tumor suppressor microRNA. Here, we found that in glioma cell lines and clinical samples, the expression of miR-1297 is significantly reduced, and re-expression of miR-1297 inhibits the growth of glioma cells both in vitro and in vivo, supporting that the notion that miR-1297 functions as a tumor suppressor microRNA in gliomas.
In our study, we demonstrated that miR-1297 inhibits the expression of HMGA1. HMGA1 is one of the members of the HMGA protein family, which are typically highly expressed during development, where several studies assign them important roles in regulating normal cell proliferation, embryonic cell growth and cell differentiation [27-29]. The expression of HMGA family proteins is barely detectable in adult tissues [22]. In many types of human cancers, the expression of HMGA1 is activated [28, 30], but its role and expression in the regulation in gliomas are still poorly understood. We demonstrated that HMGA1 promotes the growth and inhibits the apoptosis of glioma cells and that HMGA1 is negatively regulated by miR-1297. Our findings further support the concept that HMGA1 participates in the process of carcinogenesis and provide a new mechanism explaining its dysregulation in cancers.

Overall, we report for the first time that miR-1297 participates in gliomagenesis by regulating the expression of HMGA1. This may shed new light on the molecular mechanisms of gliomas and may provide a new therapeutic target for glioma prevention and treatment.

Disclosure of conflict of interest

None.

Address correspondence to: Jiannong Zhao, Department of Neurosurgery, The People’s Hospital of Hainan Province, No. 19, Xiyuing Road, Xiyuing, Haikou 570311, Hainan, China. Tel: +86-0898-66225933; Fax: +86-0898-66225933; E-mail: zhaojiannongdoc@163.com; Jian Wu, Department of Laboratory Medicine, The First People’s Hospital of Yancheng City, Yancheng 224005, Jiangsu, China. E-mail: wujianglinxing@163.com; Jianning Zhang, Tianjin Neurological Institute, Department of Neurosurgery, General Hospital, Tianjin Medical University, Tianjin 300052, China. E-mail: jianningzhang@hotmail.com

References

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