HOXA-AS2 Promotes Glioblastoma Carcinogenesis by Targeting miR-885-5p Thus Releasing RBBP4 mRNA

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Abstract

Background: HOXA-AS2, a lncRNA, had been proved to exert the promoting effect on glioblastoma, but its regulatory mechanism was not fully revealed. Our study focused on the interaction and role of HOXA-AS2/miR-885-5p/RBBP4 axis in the development of glioblastoma.

Methods: qRT-PCR was used to detect the expression of lncRNA, miRNA and mRNA in glioblastoma tissues and cells. Dual luciferase assay, RIP assay and RNA pull-down assay were applied to reveal the interaction among HOXA-AS2, miR-885-5p and RBBP4. The cell functional experiments such as CCK-8, BrdU assay and cell adhesion etc. were used to analyze the effect of HOXA-AS2/miR-885-5p/RBBP4 axis on glioblastoma cells.

Results: HOXA-AS2 and RBBP4 were overexpressed in 33 glioblastoma tissue samples and glioblastoma cells, as well as HOXA-AS2 and RBBP4 contributed to the tumorigenesis of glioblastoma cells. On the other hand, miR-885-5p was proved to be down-regulated in 33 glioblastoma tissue samples and glioblastoma cells, and it was an obstacle to the malignant phenotypes of glioblastoma cells. Meanwhile, HOXA-AS2 could negative regulate miR-885-5p, thereby promoting the RBBP4 expression since RBBP4 was targeted by miR-885-5p. Finally, HOXA-AS2 was proved to act as the positive role in glioblastoma cells via negative regulating miR-885-5p to release RBBP4.

Conclusion: HOXA-AS2 promotes the tumorigenesis of glioblastoma through targeting miR-885-5p to induce the RBBP4 expression.

Background

Glioblastoma that occurs in the nervous system under the intracranial cortex, is the most aggressive and lethal brain tumor in humans [1]. Due to the complexity of the nervous system and the limitations of surgery, glioblastoma is difficult to completely remove the tumor, which leads to high mortality [2]. In addition, the blood-brain barrier prevents chemicals from entering the brain, which limits the effective treatment of glioblastoma cells, resulting in low survival rates, high relapse rates, and poor prognosis for glioblastoma patients [3]. Although the advanced surgical treatments, traditional radiotherapy and chemotherapy have effectively improved the treatment outcomes of glioblastoma patients [4-7], investigating the underlying pathogenesis of glioblastoma is still crucial to find new diagnoses and treatments for better improving the survival of glioblastoma patients.

Long noncoding RNAs (lncRNAs) are usually defined as noncoding RNA molecules that are longer than 200 nucleotides. HOXA cluster antisense RNA 2 (HOXA-AS2) is a long non-coding RNA directly involved in biological processes in cells after being transcribed without translation. Over the past 20 years, research has shown that HOXA-AS2 has been linked to human carcinogenesis [8]. HOXA-AS2 was described to play a carcinogenic role in the pathophysiology of various cancers including pancreatic cancer, non-small cell lung cancer and osteosarcoma [9-11]. Besides, it was showed that HOXA-AS2 was up-regulated during colorectal cancer [12]. In glioma, HOXA-AS2 expression was proved to be upregulated, and silencing
HOXA-AS2 exerted the inhibitory effects on glioma cells so that HOXA-AS2 was defined as a regulator that promoted glioma [13]. Due to the complexity of the regulatory network of HOXA-AS2, our study aims to further reveal the mechanism of HOXA-AS2 in glioblastoma.

miRNAs are small non-coding RNAs encoded by endogenous genes. miRNAs mainly cause transcriptional repression or RNA degradation by binding to target genes, which leads to expression repression. Studies on miR-885 so far have shown that miRNA is involved in the biological processes of many cancers [14]. In addition, the differential expression of miR-885 provides novel therapeutic strategies and diagnostic biomarkers for cancers [15, 16]. Most of the literature retrieved about miR-885 is about miR-885-5p. It was displayed that miR-885-5p played different roles in different cancers. For instance, it was defined as a tumor-promoting factor in hepatocellular carcinoma, but as a tumor suppressor in colorectal cancer [17, 18]. In glioma, miR-885-5p expression was reduced, as well as miR-885-5p overexpression impeded cell invasion in glioma cells [19, 20]. However, the upstream regulator of miR-885-5p in glioblastoma has not been fully investigated.

RBBP4 gene is located on chromosome 1p35.1 and consists of 13 exons. This gene encodes a nuclear protein RBBP4 that belongs to a highly conserved subfamily of WD repeat proteins. The role of RBBP4 in cancer has been explored in the past decade. RBBP4 was found as a promoting regulator during gastric carcinogenesis [21]. On the other hand, RBBP4 up-regulation appeared in the hepatic metastasis of colon cancer, which provided a monitoring molecular basis for the diagnosis of the disease [22]. Silencing RBBP4, one of the NURF complex subunit, attenuated GC cell growth and increased apoptosis [21]. However, it is unclear whether RBBP4 plays a role in glioblastoma and whether RBBP4 can be regulated by miRNAs. Therefore, our study is dedicated to revealing the role of HOXA-AS2/miR-885-5p/RBBP4 axis in glioblastoma, which may help to understand the pathological processes involved in the occurrence of glioblastoma to provide evidence for its treatment.

Materials And Methods

Bioinformatics analysis

miRDB was an online tool for prediction the target genes of miR-885-5p. The upregulated DEGs were screened out by GEPIA with log2|FC|>2 and adjusted P<0.01. Then, Venny 2.1.0 was performed to overlap the common genes from miRDB and GEPIA. Finally, the common genes screened out were uploaded to String (https://string-db.org) for protein-protein interactions analysis to identify the key gene.

Sample acquisition and cell culture

Glioblastoma tissues and corresponding adjacent normal tissues from 33 patients were collected from The Fifth Affiliated Hospital of Zhengzhou University. The characteristics of 32 patients with glioblastoma were list in Table 1. Our study was approved by the Ethics Committee of The Fifth Affiliated Hospital of Zhengzhou University. Glioblastoma cell lines including U251, U87, A172, SHG44 and SNB19 and normal human astrocytes cell line (NHA) were obtained from the BNCC (Beijing, China). U251, U87,
A172 and SNB19 cells were cultured in DMEM-H (Cat#: E600004, Sangon, China) with 10% fetal bovine serum and 100 U/mL streptomycin under 5% CO$_2$ at 37 °C. As for SHG44 cell line, it was cultured in RPMI-1640 (Cat#: E600028, Sangon, China) with same culture conditions as other cell lines.

**Cell transfection**

The small interfering RNAs (siRNAs) of HOXA-AS2 (si-HOXA-AS2) and RBBP4 (si-RBBP4), miR-885-5p mimic, miR-885-5p inhibitor and negative control (NC) were synthesized and provided by GenePharma (Shanghai, China). The cells were transfected with 50 nM si-HOXA-AS2, si-RBBP4, miR-885-5p mimic and miR-885-5p inhibitor using Lipofectamine 2000 (Cat#: 11668019, Thermo Fisher Scientific, USA) at room temperature for 4 hours. After incubation for 2 days at 37°C, the transfected cells were collected to detect the transfection efficiency by qRT-PCR.

**qRT-PCR**

The total RNA from 33 clinical tumor tissue samples and corresponding non-tumor tissue samples, or cells was separated using Trizol reagent (Cat#: 15596026, Thermo Fisher Scientific, USA) according to the instructions. Then, the isolated RNA was reverse transcribed into cDNA after detection of RNA concentration. miRVana qRT-PCR miRNA Detection Kit (Cat#: AM1558, Thermo Fisher Scientific, USA) was obtained to reverse transcribe miRNA into cDNA following the protocols. As for lncRNA and mRNA, the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Cat#: 11752050, Thermo Fisher Scientific, USA) was used. After the synthesis of cDNA, the qRT-PCR was performed using StepOnePlus Real-Time PCR System (Cat#: 4376600, Thermo Fisher Scientific, USA). The data of qRT-PCR were analyzed with $2^{-\Delta\Delta Ct}$ method. GAPDH was used as the internal control of mRNA and lncRNA, and U6 as the miRNA internal control. The sequences of primers were list in Table 2.

**Subcellular fractionation location**

PARIS Kit (Cat#: AM1921, Thermo Fisher Scientific, USA) was used to separated nuclear and cytoplasmic RNA. The isolated RNA products were analyzed by qRT-PCR. The GAPDH served as cytoplasmic control and U2 was used as nuclear control.

**CCK-8 assay**

The effect of HOXA-AS2/miR-885-5p/RBBP4 axis on cell viability was assessed by CCK-8 assay. Briefly, 100 μL transfected U87 and U251 cells in a logarithmic growth phase were seeded into 96-well plates at the density of 2000 cells/well for incubation at 37 °C. After incubation for an appropriate time (0, 24, 48 and 72 hours), 10 μL CCK-8 solution was added into each well to incubate cells in incubator for 2 hours based on the manual of CCK-8 kit (Cat#: E606335, Sangon, China). The absorbance was measured at 450 nm to assess the cell viability.

**BrdU assay**
BrdU Cell Proliferation ELISA Kit (colorimetric) (Cat#: ab126556) was purchased from Abcam (UK) for BrdU assay. Briefly, $1 \times 10^5$ transfected cells was added to each well of 96-well plates. Then, 20 μL BrdU was added to each well of 96-well plates for 24 hours incubation. After incubation, the cells were fixed using 200 μL/well fixing solution for 30 minutes. Removing the fixing solution, the cells were incubated with 100 μL/well anti-BrdU antibody for 1 hour incubation at room temperature. Next, 100 μL/well Peroxidase Goat anti-mouse IgG was added into the cells for 30 minutes incubation at room temperature. Finally, the absorbance was measured at 450 nm to assess the cell proliferation ability.

**Cell adhesion assay**

10 μg/mL type I collagen or 10% bovine serum albumin was used to pre-coat 96-well plates overnight. Next, $5 \times 10^3$ cells/well transfected cells were added into the pre-coated 96-well plates for 1 hours incubation. After incubation, the non-adherent cells were removed and the adherent cells were incubated with medium for recovery. Finally, 10 μL MTT was added to cells for additional 2 hours incubation, and the absorbance was measured at 570 nm for the assessment of cell adhesion.

**The detection of cell apoptosis by flow cytometry**

The cells after transfection were resuspended in pre-chilled PBS, and diluted to $1 \times 10^6$ cells/ml using 1× Annexin binding buffer. Then, 5 μL of Annexin V-FITC staining solution and 5 μL of 100 μg/ml PI staining solution were added to the cells for 30 minutes incubation in the dark. Finally, the apoptosis rate was detected by flow cytometry according to the flow cytometer manufacturer’s instructions.

**Dual luciferase assay**

The segments of wild-type HOXA-AS2 and RBBP4 3’UTR containing the binding site of miR-885-5p and the segments of mutant HOXA-AS2 and RBBP4 3’UTR without the binding site of miR-885-5p were inserted into pmirGLO vectors. The corresponding recombinant vectors were pmirGLO-WT-HOXA-AS2, pmirGLO-MUT-HOXA-AS2, pmirGLO-WT-RBBP4 and pmirGLO-WT-RBBP4. Then, these recombinant vectors were co-transfected with miR-885-5p mimic or NC into U87 and U251 cells. After 48 hours transfection, the luciferase activity was assessed by dual-luciferase reporter assay system (Promega, USA).

**RNA immunoprecipitation (RIP) assay**

The interaction between HOXA-AS2 and miR-885-5p was further identified by RIP assay. Briefly, the U87 and U251 cells with transfection of miR-885-5p mimic were cultured for 48 hours. Then, the transfected cells were collected using trypsin, and lyzed using RNA lysis buffer. After the cells were lysed, cell lysates were incubated with magnetic beads conjugated to anti-Argonaute2 (AGO2) or IgG1 as a negative control. After washing the unbound material on the magnetic beads with RIP buffer and PBS, the magnetic beads were resuspended with TRIzol reagent (1 ml) according to the instructions to purify the bound RNA. The extracted RNAs was analyzed by qRT-PCR.

**RNA pull-down assay**
The interaction between RBBP4 and miR-885-5p was identified by RNA pull-down assay. Briefly, the $6 \times 10^5$/well cells were plated in 6-well plates for incubation overnight. Then, the biotinylated negative control (Bio-NC) and biotinylated miR-885-5p mimic (Bio-miR-885-5p) provided from RiboBio (China) were transfected the cells using lipofectamine 2000. After 48 hours, the cells were incubated with streptavidin beads for three hours. Finally, the qRT-PCR was applied to measure the DOCK1 expression that were pulled down by breads.

**Western blot assay**

Total proteins were extracted by RIPA lysis (Cat#: C500005, Sangon, China). Proteins were separated by 10% SDS-PAGE gel electrophoresis and transferred to PVDF membranes (Cat#: F019532, Sangon, China). After blocking the membranes in 5% skim milk, the hybridization membrane was incubated with primary antibodies RBBP4 (Cat#: D154089, Sangon, China) and GADPH (Cat#: D190090, Sangon, China) at 4 °C overnight. The membranes were continued to incubate with the corresponding secondary antibody for 2 hours, and the protein was detected using ECL chemiluminescence kit (Cat#: C510043, Sangon, China) according to the reagent instructions.

**Statistical analysis**

The data were represented as means ± standard deviations from three independent experimental analyses. Student’s $t$-test was used for statistical analysis between two groups, while One-factor analysis of variance (ANOVA) test was used for statistical analysis among multiple groups. P< 0.05 was considered to be statistically significant.

**Results**

**The identification of HOXA-AS2/miR-885-5p/RBBP4 interactome as the study object**

HOXA-AS2 has been confirmed to promote the aggression of glioma. We intended to expand the interacting network that involves HOXA-AS2 in glioblastoma. By interrogating the predicted 24 downstream miRNAs from ENCORE starbase database, we found that miR-885-5p, the most highly scored miRNA, was once confirmed to be a suppressor in glioma, using U87 and U251 cell line as study models [19, 20]. To identify the downstream effectors of miR-885-5p, we intersected the predicted target genes from miRDB.org database and the differentially expressed genes (DEGs) from GEPIA using the criteria of $\log_2|FC|>2$ and adjusted P<0.01. The intersected 26 genes (shown in Figure 1A) were uploaded to STRING to analyze the potential protein-protein interaction among them. As shown in Figure 1B, RBBP4 showed a very high confidence level and connected with three other genes. Additionally, RBBP4 disruption suppressed glioblastoma growth in vivo [23], and was once confirmed to be a tumor promoter in gastric cancer [21]. The specific mechanism remains unknown and the upstream regulators of RBBP4 remain unraveled. We herein identified a novel interactome in glioblastoma, and the effects of this interactome in glioblastoma has never been studied in glioblastoma before.
Si-HOXA-AS2 inhibited glioblastoma cell proliferation, migration and invasion while induced apoptosis

By using qRT-PCR, we first determined HOXA-AS2 levels in glioblastoma tissues and cells. The results showed that HOXA-AS2 expression was up-regulated by 2-fold in glioblastoma tissues compared to healthy adjacent tissues (Figure 2A). In addition, HOXA-AS2 expression in glioblastoma cell lines (U251, U87, A172, SHG44 and SNB19) was higher than that in normal cell line (NHA) (Figure 2B). Among the glioblastoma cell lines of all experimental groups, U87 and U251 showed the highest HOXA-AS2 expression, so these two cell lines were selected for subsequent biological function experiments. To further characterize HOXA-AS2, we explored the distribution of HOXA-AS2 in U87 and U251 cells. According to the experimental results of the subcellular fractionation location assay, HOXA-AS2 was located in the cytoplasm with a low content in the nucleus (Figure 2C). To investigate the effect of HOXA-AS2 on glioblastoma cells, we transfected si-HOXA-AS2 into U87 and U251 cells. qRT-PCR data showed that HOXA-AS2 silencing significantly reduced HOXA-AS2 expression by 70% compared to the blank group (Figure 2D), indicating that si-HOXA-AS2 was successfully transfected into U87 and U251 cells. From the results of the CCK-8 assay, it could be seen that compared with the blank group, HOXA-AS2 silence markedly inhibited the cell viability after the U87 and U251 cells with the transfection of si-HOXA-AS2 for 48 and 72 hours (Figure 2E). The BrdU assay proved that the proliferation ability of glioblastoma cells was impaired by si-HOXA-AS2 (Figure 2F). Similar to cell proliferation, the cell adhesion ability was also declined in glioblastoma cells with transfection of si-HOXA-AS2 (Figure 2G). Besides, silencing HOXA-AS2 increased the cell apoptosis rate by almost 12-fold in U87 cells and almost 15-fold in U251 cells (Figure 2H).

**HOXA-AS2 directly reduced miR-885-5p expression through targeted inhibition**

To explore how HOXA-AS2 promoted the pathological progress of glioblastoma, we used starBase 3.0 (http://starbase.sysu.edu.cn/index.php) to predict the miRNAs that HOXA-AS2 could bind to. The results showed that there was a binding site between miR-885-5p and HOXA-AS2 (Figure 3A). We then performed Dual luciferase assay and RIP assay to verify that HOXA-AS2 could bind to miR-885-5p. The result of Dual luciferase assay showed that miR-885-5p mimic significantly reduced the fluorescence intensity of HOXA-AS2 wild-type but had no significant effect on the fluorescence intensity of HOXA-AS2 mutant (Figure 3B). The RIP analysis also showed that there was an interaction between HOXA-AS2 and miR-885-5p (Figure 3C). Subsequent determination of miR-885-5p in collected glioblastoma tissue by qRT-PCR showed that miR-885-5p was down-regulated by 50% in glioblastoma tissues (Figure 3D), and miR-885-5p was negatively correlated with HOXA-AS2 in glioblastoma tissues (Figure 3E). In glioblastoma cells, miR-885-5p expression was also down-regulated by approximately 50% in U87 and U251 cells (Figure 3G).

**MiR-885-5p as a target of HOXA-AS2 suppressed the malignant phenotype of glioblastoma cells**

To investigate the effect of miR-885-5p down-regulated by HOXA-AS2 on the malignant phenotype of glioblastoma cells, we transfected si-HOXA-AS2, miR-885-5p inhibitor and miR-885-5p inhibitor plus si-HOXA-AS2 into U87 and U251 cells. As shown in Figure 4A, si-HOXA-AS2 led to 70% decrease of HOXA-
AS2 expression and almost 2-fold increase of miR-885-5p expression in U87 and U251 cells. The miR-885-5p inhibitor induced the 70% decrease of miR-885-5p expression, but it did not affect the HOXA-AS2 expression in glioblastoma cells. According to CCK-8 assay, miR-885-5p down-regulation promoted cell viability, but its effect on cell viability could be repressed by HOXA-AS2 silencing (Figure 4B). BrdU assay displayed that the cell proliferation ability was elevated by almost 1.5-fold in the glioblastoma cells with the transfection of miR-885-5p inhibitor, while si-HOXA-AS2 could relieve the influence of miR-885-5p inhibitor on cell proliferation (Figure 4C). The cell adhesion assay showed the same trend as the BrdU assay, showing that the promotion effect of miR-885-5p inhibitor on cell adhesion was repressed by si-HOXA-AS2 (Figure 4D). In contrast, down-regulation of miR-885-5p increased cell apoptosis rate by 2.5-fold in U87 and U251, and HOXA-AS2 silence attenuated the promoting function of miR-885-5p inhibitor in cell apoptosis (Figure 4F).

**MiR-885-5p combination of RBBP4 3'UTR to target RBBP4**

TargetScan Human 7.2 was also used to look for the target genes of miR-885-5p, finding that the position 1713-1720 of RBBP4 3'UTR was the binding site of miR-885-5p (Figure 5A). The result from Dual luciferase assay demonstrated that miR-885-5p decreased 60% fluorescence intensity of RBBP4 wild-type plasmid without affecting the RBBP4 mutant plasmid (Figure 5B). RNA pull-down assay also showed that there was an interaction between RBBP4 and miR-885-5p (Figure 5C). Then, we analyzed the expression of RBBP4 mRNA in 33 glioblastoma tissues. The RBBP4 expression was elevated by 2.5-fold in glioblastoma tissues (Figure 5D), whose expression was negatively related to miR-885-5p expression in glioblastoma tissues (Figure 5E). In glioblastoma cells, the expression of RBBP4 mRNA and protein was up-regulated in U87 and U251 cells (Figure 5F-G). At the same time, qRT-PCR data showed that miR-885-5p inhibitor led to approximately 2-fold increase of RBBP4 expression (Figure 5H). Similar to the result of mRNA, the protein level of RBBP4 in miR-885-5p inhibitor group increased by 1.5-fold in U87 cells and 1.9-fold in U251 cells (Figure 5I).

**The inhibitory effect of miR-885-5p on glioblastoma cells via down-regulating RBBP4**

To investigate whether the miR-885-5p/RBBP4 axis could regulate the tumorigenesis of glioblastoma cells, we transfected si-RBBP4, miR-885-5p inhibitor or miR-885-5p inhibitor plus si-RBBP4 into U87 and U251 cells. Western blot showed that miR-885-5p inhibitor increased the protein level of RBBP4 by more than 1.5-fold, while si-RBBP4 reduced the protein level of RBBP4 by approximately 50% (Figure 6A). The protein level of RBBP4 in miR-885-5p inhibitor plus si-RBBP4 did not show significant difference compared to blank group. The CCK-8 assay proved that si-RBBP4 inhibited the cell viability in the glioblastoma cells after 48 and 72 hours transfection, at the same time, the promotion effect of miR-885-5p inhibitor on cell viability could be relieved by si-RBBP4 (Figure 6B). The result of BrdU assay was consistent with the result of CCK-8 assay, showing that the si-RBBP4 played the inhibitory role in cell proliferation, and the promotion effect of miR-885-5p inhibitor on cell proliferation was also eliminated by si-RBBP4 (Figure 6C). The cell adhesion assay showed that si-RBBP4 resulted in approximately 30% decrease of cell adhesion compared to blank group, as well as si-RBBP4 could relieve the positive role of
miR-885-5p inhibitor in adhesion of glioblastoma cells (Figure 6D). Moreover, flow cytometry analysis showed that the 2.5-fold increase of apoptosis rate caused by RBBP4 silencing was observed both in U87 and U251 cells, and the inhibitory cell apoptosis rate caused by miR-885-5p inhibitor could be overturned by si-RBBP4 (Figure 6E).

Discussion

In this study, HOXA-AS2 was proved to be overexpressed in glioblastoma tissues and cells, and promoted glioblastoma cell proliferation, migration and invasion while inhibiting apoptosis. On the other hand, miR-885-5p, which was directly inhibited by HOXA-AS2, was down-regulated during the pathological process of glioblastoma, and repressed the aggressiveness of glioblastoma cells. Finally, RBBP4 as a measurable downstream target of miR-885-5p was found up-regulated in glioblastoma and enhanced the malignant phenotypes of glioblastoma cells. Overall, HOXA-AS2 promoted glioblastoma through the HOXA-AS2/miR-885-5p/RBBP4 axis.

HOXA-AS2, a lncRNA, was found to be up-regulated and exert the promoting function in cancer processes. For instance, HOXA-AS2 has been shown to stimulate osteosarcoma cell proliferation, migration and invasion [11]. Several studies described HOXA-AS2 as an oncogene that promoted the malignant proliferation of non-small cell lung cancer cells [24-26]. Similarly, HOXA-AS2 was indicated to advance bladder cancer by directly regulating the expression of miR-125b, thereby regulating Smad2 [27]. Furthermore, HOXA-AS2 was identified to promote breast cancer by HOXA-AS2/miR-106a/SCN3 axis [28]. In glioma cells, HOXA-AS2 recruited EZH2 to up-regulate RND3 to promote the generation of glioma cells, thereby accelerating tumor growth [13]. Correspondingly, our study showed that HOXA-AS2 up-regulation occurred in glioblastoma tissues, thereby promoting the aggressiveness of glioblastoma cells. Different from the previous study on HOXA-AS2 in glioma, our study showed that HOXA-AS2, as an oncogene in glioblastoma, increased RBBP4 through targeting miR-885-5p to enhance cancer cell viability.

MiR-885-5p had been proved to exerted the different function in different cancers due to its abnormal expression in multiple cancer types. For instance, miR-885-5p inhibited hepatocellular carcinoma cell proliferation, invasion and angiogenesis by inhibiting the expression of AEG1 [29]. In gastric cancer, the miR-885-5p expression increased in gastric cancer cells, and the cell functional experiments proved that downregulation of miR-885-5p inhibited gastric cancer cell proliferation, colony formation and invasion [30]. In glioblastoma, Yan et al. used the miRNA microarray in 60 glioblastoma multiforme samples, finding that miR-885-5p was high correlation with MMP-9 expression [20]. What’s more, in the study from Yan et al., miR-885-5p overexpression could reduce the MMP-9 level that was a oncogene in glioblastoma, thereby suppressing glioma cell invasion. Anther study also found that miR-885-5p overexpression exerted the tumor suppressive effects on gliomagenesis [19]. In our study, miR-885-5p was predicted as the downstream miRNA of HOXA-AS2 so that miR-885-5p attracted our attention. By a series of cytological experiments such as CCK-8, BrdU, cell adhesion assay etc., our research showed that miR-885-5p inhibited the malignant phenotypes of glioblastoma cells, which was consistent with the previous studies on miR-885-5p in glioma cells.
It was reported that RBBP4, as a subunit of NURF complex, facilitated hepatocellular carcinoma by interacting with other components to silence tumor suppressor genes [31]. In another study, RBBP4 silencing impaired the proliferation of gastric cancer cells but stimulated apoptosis [21]. These findings provided RBBP4 might be a oncogene for the progress of cancer. In our study, RBBP4 was identified as the key gene in glioblastoma by bioinformatics analysis. By Dual luciferase assay and RNA pull-down assay, RBBP4 was confirmed as the target gene of miR-885-5p that could suppress the RBBP4 expression. Besides, the RBBP4 served as an oncogene to facilitate malignant phenotypes of glioblastoma cells, and it could overturn the inhibitory influence of miR-885-5p on glioblastoma cells.

Our study proved the function of HOXA-AS2/miR-885-5p/RBBP4 axis in glioblastoma cells. However, we have not further study the downstream signaling pathway of RBBP4 to fully reveal the mechanism of HOXA-AS2/miR-885-5p/RBBP4 axis on the progression of glioblastoma. Besides, animal experiments to further study the promoting effects of HOXA-AS2 are also needed, which is our next research plan.

**Conclusion**

Our research for the first time revealed the influence of HOXA-AS2/miR-885-5p/RBBP4 axis on glioblastoma cells carcinogenesis. In particular, HOXA-AS2 contributed to the glioblastoma cell viability, proliferation and adhesion, but it inhibited cell apoptosis by negative regulating miR-885-5p to release RBBP4. Our findings may help to provide the theoretical basis and targets for the treatment of glioblastoma.

**Abbreviations**

miRNAs: microRNAs

PBS: phosphate buffered solution

EdU: 5-ethynyl-2'-deoxyuridine

HOXA-AS2: HOXA cluster antisense RNA 2

qPCR: quantitative real-time PCR

**Declarations**

**Ethics approval and consent to participate**

Ethic Committee of T The Fifth Affiliated Hospital of Zhengzhou University (Zhengzhou, China) approved the study.

**Consent for publication**
Informed consent was obtained from all patients.

Availability of Data and Materials

The datasets used during the current study are available from the corresponding author on reasonable request.

Funding

This research has received no funds.

Competing interests

There is no conflict of interest existed among the authors.

Authors’ contributions

JXS designed the experiments. SC and HDG conducted the experiments. BBW and HBG wrote the manuscript.

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Tables
Table 1 Baseline characteristics of 33 patients with glioblastoma

| Total no. patients = 33 | No. (%) |
|-------------------------|---------|
| Age at diagnosis (years)|         |
| Age >55                 | 18 (54.55) |
| Age ≤55                 | 15 (45.45) |
| Gender                  |         |
| Female                  | 20 (60.61) |
| Male                    | 13 (39.39) |
| Tumor origin            |         |
| Primary                 | 23 (69.7) |
| Secondary               | 10 (30.3) |
| Tumor Localization      |         |
| Left hemisphere         | 11 (33.33) |
| Right hemisphere        | 14 (42.43) |
| Both hemisphere         | 8 (24.24) |
| Tumor volume (median, range) | 17.9 cm$^3$ (1.2-57.8) |

Table 2. Primer sequences for RT-qPCR

| Gene name | Primer-F (5’-3’) | Primer-R (5’-3’) |
|-----------|------------------|------------------|
| HOXA-AS2  | AACCCATCTTTGCCTTCTGC | CGGAGGAGTTTGGAGTTGG |
| miR-885-5p| GTCCATTACACTACCTGCCTC | CGCGAGCACAGAATTAATACG |
| U6        | CTCGCTTCGCGACGACA | AACGCTTCACGAATTTCGT |
| RBBP4     | GTAGAGAGCTCTTCAGCAAGAC | AGGAACAGCTGGAGGAAATG |
| GAPDH     | GGGAGCAGAAAAAGGGTCAT | GAGTCCTCCACGATACCAA |
Figure 1

RBBP4 was identified as our interested gene to be investigate in glioblastoma. (A) The 26 genes were overlapped from miRDB and GEPIA by Venny 2.1.0. miRDB was an online tool to predict the target genes of miR-885-5p. GEPIA was a online tool to select the DEGs involving glioblastoma with log2|FC|>2 and adjusted P<0.01. (B) RBBP4 was the key gene connecting three other genes by String analysis. String was an online tool to analyze the potential protein-protein interaction of 26 genes.
Si-HOXA-AS2 inhibited cell viability, cell proliferation and cell adhesion but induced cell apoptosis in glioblastoma. (A) HOXA-AS2 expression in glioblastoma tissues and non-tumor tissues was analyzed by qRT-PCR. N=33. (B) HOXA-AS2 expression in glioblastoma cell lines (U251, U87, A172, SHG44 and SNB19) and normal human astrocytes cell line (NHA) was analyzed by qRT-PCR. (C) The intracellular distribution of HOXA-AS2 was identified by subcellular fractionation location assay. (D) Transfection efficiency of si-HOXA-AS2 was verified in U87 and U251 cell lines by qRT-PCR. (E) The viability of the transfected U87 and U251 cells was measured by CCK-8 assay. (F) The proliferation of the transfected U87 and U251 cells was measured by BrdU assay. (G) The adhesion ability of the transfected U87 and U251 cells was evaluated via cell adhesion assay. (H) The apoptosis rate of the transfected U87 and U251 cells was evaluated via flow cytometry. NC, negative control. Si-LNC, si-HOXA-AS2. The cells in blank group without any treatments. The data were the means ±SD from three experiments. *P< 0.05, **P< 0.001.
HOXA-AS2 was the target gene of miR-885-5p. (A) starBase showed the binding site between HOXA-AS2 and miR-885-5p. (B) The potential binding site between HOXA-AS2 and miR-885-5p was identified by Dual luciferase assay. Mimic, miR-885-5p mimic. WT-Inc, wild-type HOXA-AS2. MUT-Inc, mutant HOXA-AS2. (C) The interaction between HOXA-AS2 and miR-885-5p was evaluated by RIP analysis. (D) MiR-885-5p expression in glioblastoma tissues and non-tumor tissues was analyzed by qRT-PCR. N=33. (E) The
correlation analysis of miR-885-5p and HOXA-AS2. (F) MiR-885-5p expression in glioblastoma cell lines (U251 and U87) and normal human astrocytes cell line (NHA) was analyzed by qRT-PCR. The data were the means ±SD from three experiments. *P< 0.05, **P< 0.001.
MiR-885-5p regulated by HOXA-AS2 could suppress the malignant phenotype of glioblastoma cells. (A) Transfection efficiency of si-HOXA-AS2 and miR-885-5p inhibitor was verified by qRT-PCR. (B) The viability of the transfected U87 and U251 cells was measured by CCK-8 assay. (C) The proliferation of the transfected U87 and U251 cells was measured by BrdU assay. (D) The adhesion ability of the transfected U87 and U251 cells was detected by cell adhesion assay. (E) The apoptosis rate of the transfected U87 and U251 cells was evaluated via flow cytometry. NC, negative control. Si-LNC, si-HOXA-AS2. Inhibitor, miR-885-5p inhibitor. The cells in blank group without any treatments. The data were the means ±SD from three experiments. *P< 0.05, **P< 0.001.
A

Predicted consequential pairing of target region (top) and miRNA (bottom)

Position 1713-1720 of RBBP4 3' UTR

5' AAAUGAAACCACCAUGUAUGGA...

3' UCUCCGGUCCAUCAUCACU

B

![Graphs showing relative luciferase activity](image)

C

![Graph showing relative expression of RBBP4](image)

D

![Scatter plot showing relative expression of RBBP4 and miR-885-5p](image)

E

![Graph showing correlation between relative expression of RBBP4 and miR-885-5p](image)

F

![Graph showing relative expression of RBBP4](image)

G

![Image showing Western blot analysis of RBBP4 and GAPDH](image)

H

![Graphs showing relative expression of miR-885-5p and RBBP4](image)
MiR-885-5p directly targeted RBBP4 by binding to its 3’UTR. (A) Potential binding site between miR-885-5p and RBBP4 was predicted by TargetScan Human 7.2. (B) Potential binding site between miR-885-5p and the 3’UTR of RBBP4 was demonstrated by Dual luciferase assay. NC, negative control. Mimic, miR-885-5p mimic. (C) The interaction between RBBP4 and miR-885-5p was evaluated by RNA pull-down assay. Bio-NC, biotinylated negative control. Bio-miR-885-5p, biotinylated miR-885-5p. (D) RBBP4 expression in glioblastoma tissues and normal tissues was detected by qRT-PCR. N=33. (E) The correlation analysis of miR-885-5p and RBBP4. (F) RBBP4 expression in glioblastoma cell lines (U251 and U87) and normal human astrocytes cell line (NHA) was evaluated by qRT-PCR. (G) The expression of RBBP4 protein in glioblastoma cell lines (U251 and U87) and normal human astrocytes cell line (NHA) was detected by western blot. (H) The expression of RBBP4 mRNA increased by miR-885-5p inhibitor in U87 and U251 cells. (I) The expression of RBBP4 protein increased by miR-885-5p inhibitor in U87 and U251 cells. NC, negative control. Inhibitor, miR-885-5p inhibitor. The cells in blank group without any treatments. The data were the means ±SD from three experiments. *P< 0.05, **P< 0.001.
Figure 6

The miR-885-5p inhibited the tumorigenesis via targeting RBBP4. (A) Transfection efficiency of miR-885-5p inhibitor and si-RBBP4 was verified by western blot assay. (B) The viability of the transfected U87 and U251 cells was measured by CCK-8 assay. (C) The proliferation of the transfected U87 and U251 cells was measured by BrdU assay. (D) The adhesion of the transfected U87 and U251 cells was detected by cell adhesion assay. (E) The apoptosis rate of the transfected U87 and U251 cells was evaluated via flow cytometry. NC, negative control. Si-RNA, si-RBBP4. Inhibitor, miR-885-5p inhibitor. The data were the means ±SD from three experiments. *P< 0.05, **P< 0.001.