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Hypoxia-induced miR-497 decreases glioma cell sensitivity to TMZ by inhibiting apoptosis

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ABSTRACT

Understanding the resistance of glioma cells to chemotherapy has been an enormous challenge. In particular, mechanisms by which tumor cells acquire resistance to chemotherapy under hypoxic conditions are not fully understood. In this study, we have found that miR-497 is overexpressed in glioma and that hypoxia can induce the expression of miR-497 at the transcriptional level by binding with the hypoxia response element in the promoter. Ectopic overexpression of miR-497 promotes chemotherapy resistance in glioma cells by targeting PDCD4, a tumor suppressor that is involved in apoptosis. In contrast, the inhibition of miR-497 enhances apoptosis and increases the sensitivity of glioma cells to TMZ. These results suggest that miR-497 is a potential molecular target for glioma therapy.

1. Introduction

Hypoxia is a common characteristic of rapidly growing tumors [1]. Hypoxic conditions induce many different types of physiological or pathophysiological responses, including high-altitude adaptation, stroke, wound healing, myocardial infarction, and cancer [2]. These changes are carefully controlled by a large number of hypoxia-inducible genes [2]. Among these genes, hypoxia-inducible factor 1α (HIF1α) is the key transcription factor induced by hypoxia, increasing the expression of many genes that are involved in metastasis, tumor recurrence, and angiogenesis [3–5]. Hypoxia also promotes tumor cell resistance to apoptosis [6]; however, the detailed mechanism is not fully understood.microRNAs are small, non-coding, single-stranded RNAs that are 20–23 nucleotides in length and that suppress gene expression in a sequence-specific manner [7]. The seed region containing two to eight bases in the 5′ portion of the miRNA sequence is important for target mRNA recognition. miRNAs suppress gene expression via complementarity between the miRNA seed region and the 3′ untranslated region (UTR) of the target mRNA [7]. Inside the primary tumor, the rapid growth of cancer cells frequently outgrows its blood supply, resulting in many tumors in regions where the oxygen concentration is significantly lower than in normal tissue. To survive in hypoxic conditions, cancer cells have altered their intrinsic gene expression patterns to inhibit apoptosis. Recent studies have shown that hypoxia induces the expression of a large number of miRNAs, termed "hypoxamirs" and that these miRNAs regulate cell proliferation, apoptosis, and metastasis [8].

Aberrant expression of miR-497 has been implicated in numerous types of cancers and appears to be significantly associated with the clinical outcome of human cancer patients. For example, the methylation status of CpG islands upstream of the miR-497 gene has been shown to downregulate miR-497 [9]. Forced overexpression of miR-497 has been shown to inhibit breast cancer cell growth and invasion [9]. In hepatocellular carcinoma, miR-497 was reported to be a tumor suppressive miRNA, showing growth-suppressive activities by targeting CDK6, CCNE1, CDC25A, and CDK4 [10].

However, it has not been clear whether miR-497 is involved in the drug resistance of glioma. In this study, we found that miR-497 is overexpressed in human glioma cancer tissue and that hypoxia can induce the expression of miR-497 at the transcriptional level by binding with the hypoxia response element in the promoter. Ectopic expression of miR-497 promotes chemotherapy resistance in glioma cells by targeting PDCD4, suggesting that miR-497 is a potential molecular target for sensitizing treatment.

2. Materials and methods

2.1. Cell culture and reagents

Glioma cells U87 and U251 and HEK293T cells were purchased from American Type Culture Collection (Manassas, VA, USA). All

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cells were cultured in DMEM medium supplemented with 10% FBS (fetal bovine serum) and antibiotics. SYBR Green PCR master mix and TaqMan microRNA reverse transcription kit were purchased from ABI (Foster, CA, USA). Antibodies against PDCD4, PARP, and cleaved PARP were purchased from Cell Signaling Technology (Boston, MA, USA). Temozolomide was purchased from Sigma. The Dual-Luciferase Reporter Assay kit was purchased from Promega (Madison, WI, USA).

2.2. Plasmids and constructs

To construct the pmirGLO-PDCD4-3′UTR-WT plasmid, a fragment of the wild-type 3′UTR of human PDCD4 mRNA (GenBank accession number: NM_014456.4) containing the putative miR-497 binding sequence was amplified and cloned into the SacI and XhoI sites of the pmirGLO-control vector (Promega). pmirGLO-PDCD4-3′UTR-MUT, which carried mutations in the sequence complementary to the seed region of miR-497, was generated based on the pmirGLO-PDCD4-3′UTR-WT plasmid by site-specific mutagenesis using the following primers: forward primer: 5-aagttaaccaggtaaaaccccatgttgggtccaggt-3; reverse primer: 5-acctggacccaacatggggtt-3.

2.3. Transfections

All transfection assays were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The miR-497 inhibitor, negative controls (scrambles), and miR-497 mimics were purchased from GenePharma (Shanghai, China). The miR-497 inhibitor and mimics were used at a final concentration of 50 nM.

2.4. Luciferase reporter assay

For the luciferase reporter assay, 293T cells were seeded in a 24-well plate and were grown to 80–90% confluence. To detect the interaction between miR-497 and PDCD4 3′UTR, cells were cotransfected with 50 nM of either scramble or miR-497 mimics and 40 ng of either pmirGLO-PDCD4-3′UTR-WT or pmirGLO-PDCD4-3′UTR-MUT using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. To detect whether the putative HRE in the miR-497 promoter was functional, 293T cells were transfected with wtHRE1, mut1HRE1, mut2HRE1, or mut3HRE1 and cultured in 1% O_2 conditions. The cells were collected 48 h after transfection and analyzed using the Dual-Luciferase Reporter Assay System. A plasmid constitutively expressing Renilla luciferase was cotransfected as an internal control to correct for differences in both transfection and harvesting efficiencies. The transfections were performed in duplicate, and at least three independent experiments were performed.

2.5. Western blotting

Cells were harvested in lysis buffer (10 mM, Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100), containing protease and phosphatase inhibitors (Roche Applied Science). Samples were incubated for 15 min on ice and centrifuged 10 min at 13 000 rpm at 4 °C. Protein concentrations were determined using the BCA reagent of cell lysate (40 μg) was separated by SDS–PAGE and transferred to a PVDF membrane (Fisher Scientific). After blocking for 60 min in TBST (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20) with 5% skim milk, membranes were incubated with the primary antibody for 1 h, washed, and incubated for 30 min with horseradish peroxidase-conjugated secondary antibody. The membranes were washed and incubated with enhanced chemiluminescence substrate (BioRad).

2.6. Quantitative RT-PCR

Total RNA was isolated from cells using Trizol Reagent (Life Technologies), and 1 μg of total RNA was reverse transcribed in a 50 μl reaction using TaqMan Reverse Transcription Reagents (Applied Biosystems Inc.) with the stem loop primers. 2 μl of the reverse transcribed product was added to the SYBR Green master mix to quantify miRNA expression. The results were normalized to RNU48.

2.7. ChIP assays

ChIP assays were performed using the ChIP Assay kit according to the manufacturer's instructions. Cells were crosslinked with 1% formaldehyde for 20 min at 37 °C and quenched in 0.125 M glycine. The immunoprecipitated DNA from the sonicated cell lysates was quantified using real-time PCR analysis with the SYBR Green system. Primer sequences used are listed as follows: HRE1-forward: 5′-tcttttcaaaataggggacc-3′; HRE1-reverse: 5′-gggctgcag tttaggggtat-3′; HRE2-forward: 5′-cctggagttgggggtgggga-3′; HRE2-reverse: 5′-cctccatcagcccatccacc-3′; NC-forward: 5′-aataagttcaa tttggagtt-3′; NC-reverse: 5′-gatacacaggaataccccac-3′.

2.8. Apoptosis assay

Cells transfected with miR-497 mimics or miR-497 inhibitor were treated with TMZ at a final concentration of 100 μM and cultured for 24 h to measure apoptosis. At the end of the incubation period, floating as well as adherent cells were harvested by trypsin and washed with PBS. Cells were stained with 5 μl annexin V and 60 μl 1× binding buffer for 15 min at room temperature in the dark, and then 120 μl 1× binding buffer and 5 μl propidium iodide. Apoptosis was measured using flow cytometry.

3. Results

3.1. miR-497 is overexpressed in glioma

First, we analyzed the miRNA expression profile from a previously published dataset (Gene expression omnibus accession GSE25631) and found that miR-497 is overexpressed in glioma (Fig. 1A) [11]. Next, we confirmed the expression of miR-497 in glioma. qRT-PCR analysis shows that among three matched sample pairs, miR-497 expression is significantly higher in clinical glioma tissue than in adjacent normal tissue (Fig. 1B). These results show that miR-497 is overexpressed in glioma.

3.2. Hypoxia induces the expression of miR-497 in glioma cells

Previous studies have shown that hypoxia induces miR-497 expression in retinoblastoma cells [12], but whether this occurs in glioma cells has not been elucidated. To detect miR-497, U251 and U87 cells were grown for 12 h in 1% oxygen, and mature miR-497 was measured using quantitative PCR in these two cell lines. The data show that the levels of mature miR-497 are significantly increased in U251 and U87 cells under hypoxic conditions (Fig. 2A and B). Because HIF1-α is a key transcription factor that mediates the induction of many hypoxia-inducible genes, we treated glioma cells with DMOG, a well-established agent that induces HIF1-α [13], to examine whether HIF1-α can upregulate miR-497 in cancer cells. The data show that DMOG increases the level of miR-497 in U251 and U87 cells compared with controls (Fig. 2Aand B). As expected, the protein level of HIF1-α increases in U251 and U87 cells treated with 1% oxygen and DMOG (Fig. 2C and D), as evaluated using Western blot analysis. In contrast, knock down of HIF1α with shRNA under

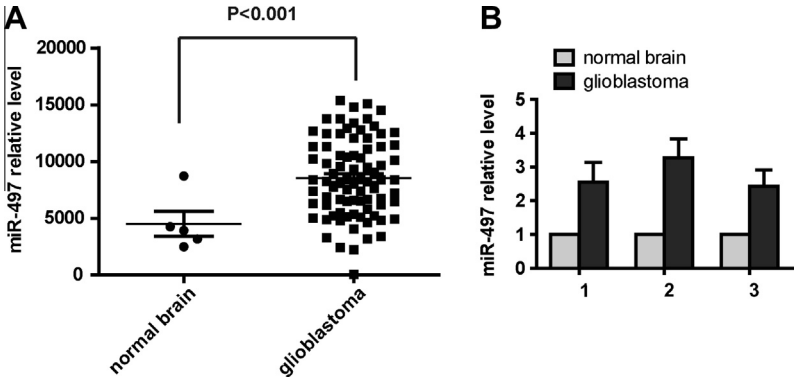

Fig. 1. miR-497 is overexpressed in glioma. (A) miR-497 is significantly overexpressed in glioma compared with normal brain in microRNA expression assays. (B) Real-time PCR analysis shows that miR-497 is significantly increased in glioma tissue compared with normal tissue.

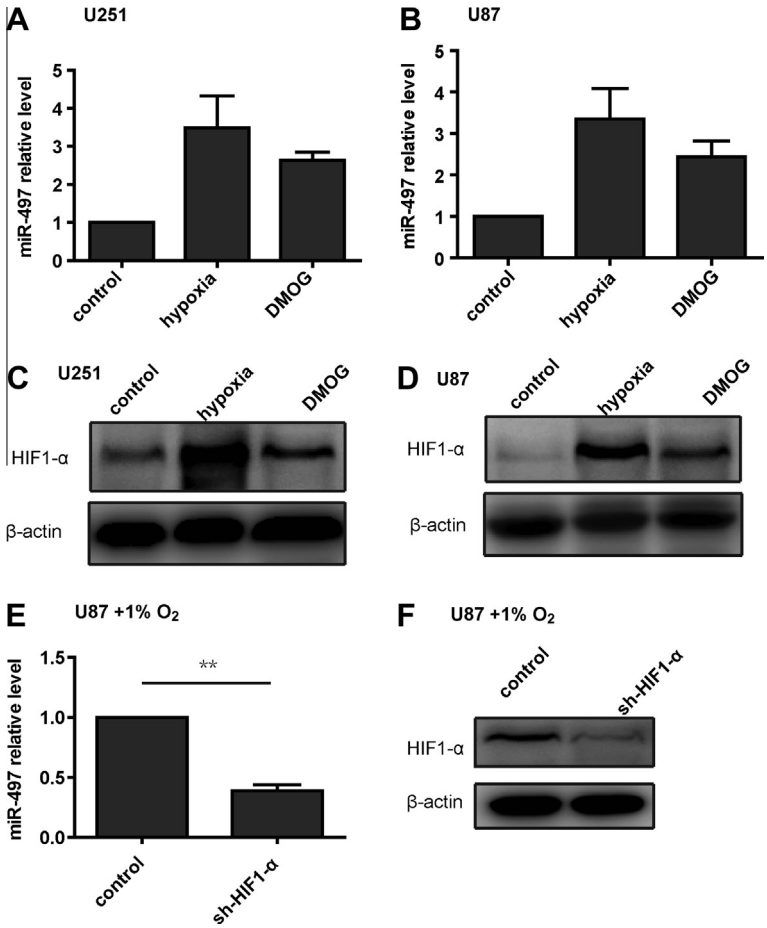

Fig. 2. Hypoxia induces the expression of miR-497 in glioma cells. (A) Quantitative PCR analysis shows that hypoxia increases the level of mature miR-497 in U251 cells. (B) Quantitative PCR analysis shows that hypoxia increases the level of mature miR-497 in U87 cells. (C) Western blot analysis shows that the protein level of HIF1α is increased in U251 cells under hypoxic conditions and DMOG treatment. (D) Western blot analysis shows that the protein level of HIF1α is increased in U87 cells under hypoxic conditions and DMOG treatment. (E) Quantitative PCR analysis shows that the miR-497 level is decreased in U87 cells treated with shRNA-HIF1α under hypoxic conditions (** represents $P < 0.01$). (F) Western blot analysis shows that the protein level of HIF1α is decreased in U87 cells treated with shRNA-HIF1α under hypoxic conditions.

hypoxic conditions decreased the miR-497 level in U87 cells (Fig. 2E and F).

3.3. HIF1-α induces the expression of miR-497 at the transcriptional level

Given that HIF1-α is a well-characterized transcription factor, we hypothesized that HIF1-α induces the expression of miR-497 at the transcriptional level. To test this hypothesis, we first examined the expression level of pri-miR-497 in cells under hypoxic conditions and DOMG treatment. The real-time PCR data show that both hypoxia and DOMG administration significantly increase pri-miR-497 levels (Fig. 3A). To further identify how HIF1-α regulates miR-497 expression, we analyzed the 3000 bp region upstream of pri-miR-497 and found that there are three hypoxia response elements (Fig. 3B). To investigate whether HIF1-α binds to the

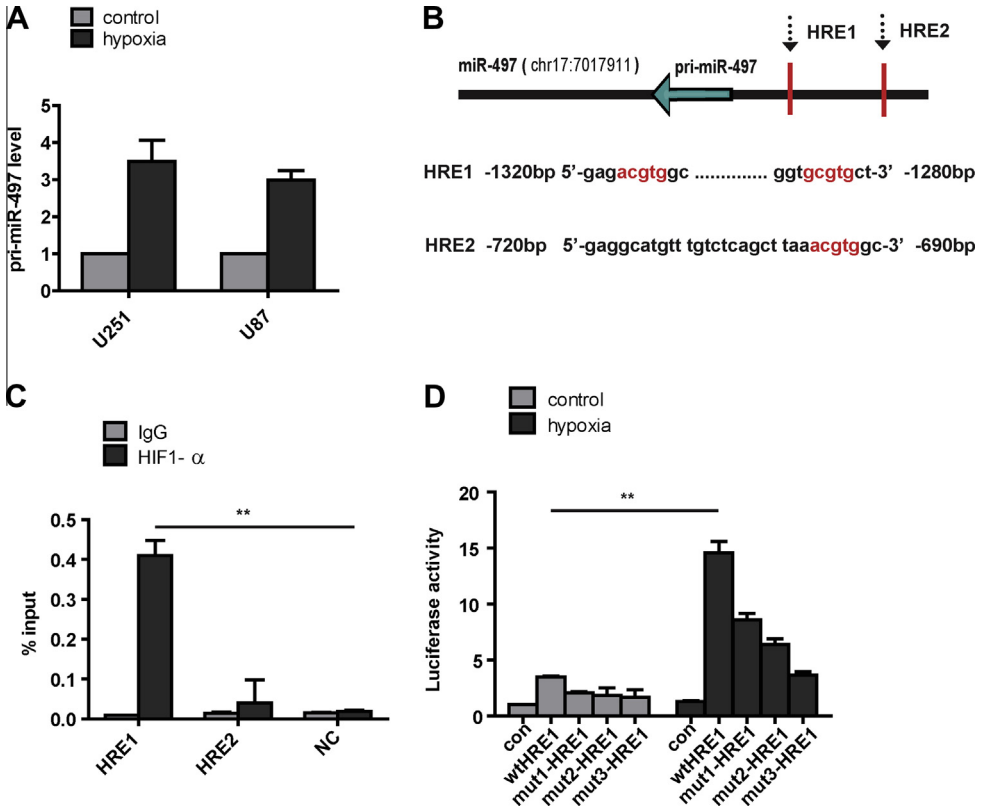

Fig. 3. HIF1α induces the expression of miR-497 at the transcriptional level. (A) Quantitative PCR analysis shows that hypoxia increases the level of pri-miR-497 in U251 and U87 cells. (B) The putative HRE sites in the promoter of pri-miR-497, HRE: hypoxia response element. (C) qChIP analysis shows that HIF1α is significantly enriched in HRE1, but not in HRE2; NC represents the negative control without the HRE sequence (** represents $P < 0.01$). (D) The luciferase reporter assay shows that miR-497 HRE1 significantly increases luciferase activity in hypoxic conditions and that mutant HRE1 inhibits the increased luciferase activity induced by hypoxia; mut1-HRE1 represents the mutation of 5′-acgtg-3′ to 5′-aaatg-3′; mut2-HRE1 represents the mutation of 5′-gcgtg-3′ to 5′-aaatg-3′; mut3-HRE1 represents both mutations in mut1-HRE1 and mut2-HRE1 (** represents $P < 0.01$).

promoter of the miR-497 gene, chromatin immunoprecipitation (ChIP) assays were performed. Our data show that HIF1-α binds to HRE1 elements in the 1.5 kb region upstream of the miR-497 promoter (Fig. 3C). However, there is no significant enrichment in HRE2 (Fig. 3C), suggesting that HIF1-α regulates miR-497 expression at the transcriptional level. To further confirm whether the putative HRE1 in the miR-497 promoter is functional, a 200 bp fragment encompassing the miR-497 promoter HRE1 was inserted into the pGL3 promoter. HEK293T cells transfected with the wild-type miR-497 promoter reporter, control reporter, mutant miR-497 promoter, or pSV-Renilla vector, were exposed to 20% O_2 or 1% O_2 for 12 h. The wild-type miR-497 promoter significantly increases Fluc activity in cells under hypoxic conditions (Fig. 3D). The mutation in mut1-HRE1 (5′-acgtg-3′ to 5′-aaatg-3′) and in mut2-HRE1 (5′-gcgtg-3′ to 5′-aaatg-3′) at the core binding site, eliminating HIF1α binding, leads to a significant decrease in hypoxia-induced Fluc activity, and the mutations in mut3-HRE1 (5′-acgtg-3′ to 5′-aaatg-3′ and 5′-gcgtg-3′ to 5′-aaatg-3′) lead to a greater decrease in Fluc activity than that observed for mut1-HRE1 or mut2-HRE1 (Fig. 3D). Taken together, these data demonstrate that HIF1α binds to the site of HRE1 in the miR-497 promoter and directly activates its transcription.

3.4. miR-497 increases glioma cell resistance to apoptosis

To explore the biological function of miR-497 in glioma cells, TMZ-induced apoptosis was first analyzed in U87 and U251 cells transfected with miR-497 mimics. Flow cytometry analysis shows that overexpression of miR-497 decreases the percentage of annexin V positives in both cell lines (Fig. 4A and B), suggesting that miR-497 overexpression suppresses TMZ-induced apoptosis. To further confirm the effects of miR-497 on apoptosis in glioma cells, TMZ-induced apoptosis was analyzed in U87 and U251 cells transfected with the miR-497 inhibitor, using tumor cells transfected with scramble oligomers as a control. Real-time PCR shows that the miR-497 inhibitor significantly decreases the level of miR-497 (data not shown). Flow cytometry analysis shows that TMZ significantly increases the percentage of annexin V positive cells in glioma cells transfected with miR-497 inhibitor (Fig. 4C and D). Western blot analysis shows that TMZ dramatically induces cleavage of PARP, a well-known marker of apoptosis; overexpression of miR-497 attenuates the level of cleaved PARP (Fig. 4E), and the knockdown of miR-497 enhances the level of cleaved PARP (Fig. 4F).

Taken together, these observations suggest that overexpression of miR-497 protects the tumor cells from apoptosis and increases tumor cell resistance to therapeutic chemicals.

3.5. miR-497 suppresses the expression of PDCD4, a tumor suppressor involved in apoptosis

To dissect the mechanism by which miR-497 increases tumor cell resistance to TMZ, the downstream targets of miR-497 were identified using the web-based software Targetscan. PDCD4 is an apoptosis-related protein that is potentially targeted by miR-497 based on the pairing of the seed sequence of miR-497 (Fig. 5C). The 3′UTR of PDCD4 was cloned into a dual-luciferase UTR vector, as shown in the schematic, and the luciferase reporter assay was performed. The results show that luciferase activity is significantly reduced by miR-497 in cells expressing wild-type PDCD4, but not

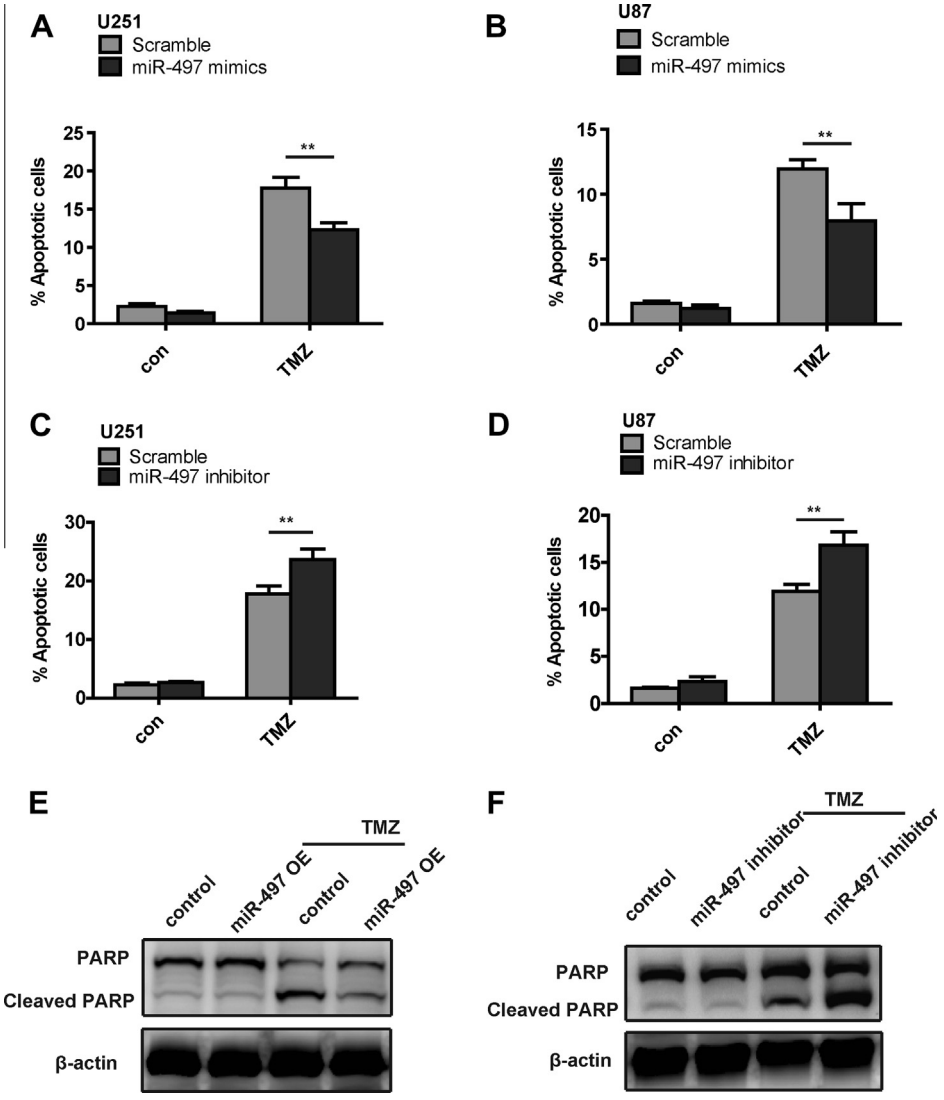

Fig. 4. miR-497 increases glioma cell resistance to apoptosis. (A) Flow cytometry analysis shows that miR-497 overexpression inhibits apoptosis induced by TMZ in U251 (** represents $P < 0.01$). (B) Flow cytometry analysis shows that miR-497 overexpression inhibits apoptosis induced by TMZ in U87 (** represents $P < 0.01$). (C) Flow cytometry analysis shows that the knockdown of miR-497 increases apoptosis induced by TMZ in U251 (** represents $P < 0.01$). (D) Flow cytometry analysis shows that the knockdown of miR-497 increases apoptosis induced by TMZ in U87 (** represents $P < 0.01$). (E) Western blot analysis shows that miR-497 overexpression decreases the cleaved PARP induced by TMZ. (F) Western blot analysis shows that the knockdown of miR-497 increases the cleaved PARP induced by TMZ.

in cells expressing mutant PDCD4 (Fig. 5D). Further, the protein level of PDCD4 was determined using Western blot analysis in U87 and U251 cells transfected with miR-497 mimics or inhibitor. The data show that overexpression of miR-497 dramatically decreases the PDCD4 protein level (Fig. 5A); in contrast, the miR-497 knockdown increases the PDCD4 protein level (Fig. 5B). Taken together, these data indicate that miR-497 suppresses the expression of PDCD4.

To detect whether PDCD4 is required for miR-497 in regulating apoptosis induced by TMZ, We knock down the expression of PDCD4 in U251 cells transfected with miR-497 inhibitor and then administrated these cells with TMZ. The results show that siRNA-PDCD4 significantly decreases the PDCD4 level in cells treated with miR-497 inhibitor (Fig. 5E). As expected, the cleavage PARP induced by TMZ is decreased in cells contransfected with miR-497 inhibitor and siRNA-PDCD4 compared with the cells transfected with miR-497 inhibitor (Fig. 5F). The data indicate that the role of miR-497 in regulating apoptosis induced by TMZ is mediated by PDCD4.

4. Discussion

Glioblastoma is one of the most common and lethal forms of cancer in the central nervous system. Surgical techniques, radiotherapy, and chemotherapy are the main treatments for glioblastoma, but they have little effect in extending the outcome. The difficulty in treating this cancer is that there are many mechanisms of drug resistance to consider, such as decreased uptake of water-soluble drugs, increased ability to repair DNA damage, reduced apoptosis, altered drug metabolism, and increased energy-dependent efflux of chemotherapeutic drugs that diminish the ability of cytotoxic agents to kill cancer cells [14]. TMZ is the first line chemotherapeutic drug which is used for the treatment of glioma, however, lower than 40% of glioma patients initially respond to therapy and resistance develops rapidly. Elucidating the mechanism under TMZ resistance in glioma is becoming more important. Recent works have underscored the involvement of microRNAs in cancer development, with several studies suggesting their involve-

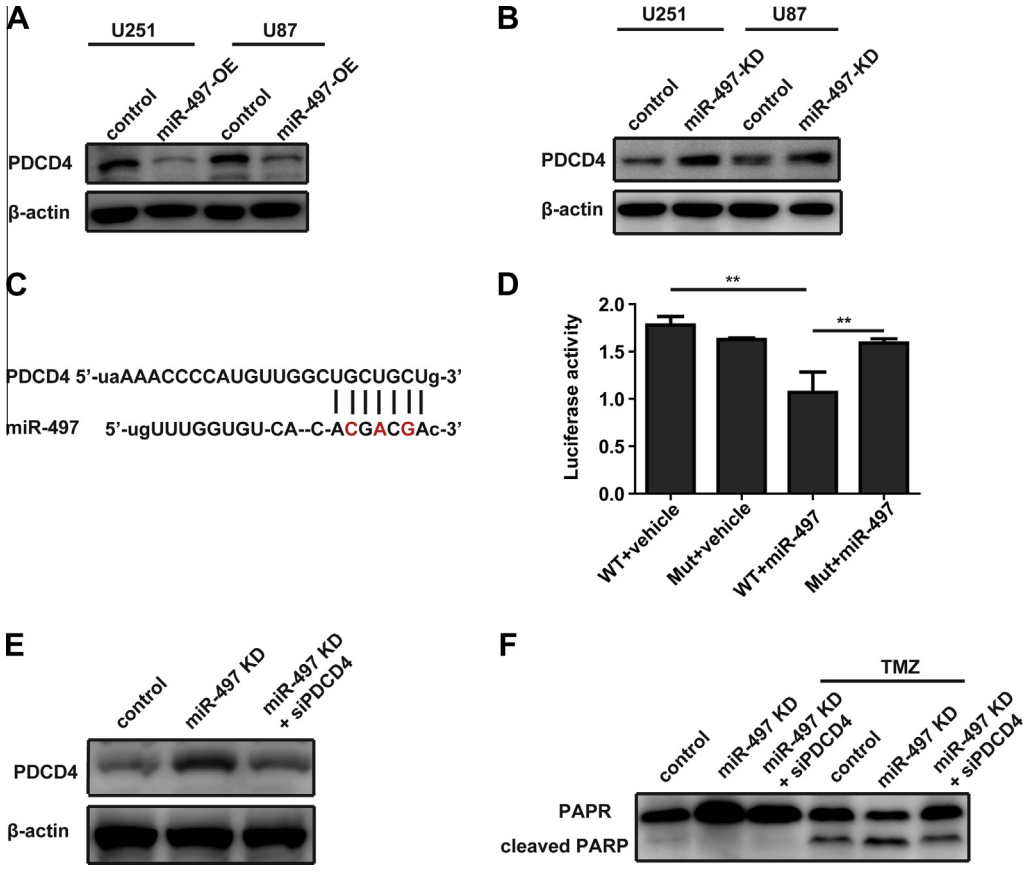

Fig. 5. miR-497 suppresses the expression of PDCD4, a tumor suppressor involved in apoptosis. (A) Western blot analysis shows that overexpression of miR-497 inhibits the protein level of PDCD4 in U251 and U87. (B) Western blot analysis shows that the knockdown of miR-497 increases the protein level of PDCD4 in U251 and U87. (C) The sequences in the 3′UTR of PDCD4 were predicted to bind to miR-497. The red nucleotides were mutated to their complementary nucleotides. (D) The luciferase reporter assay shows that miR-497 represses luciferase activity in the wild-type UTR, but does not repress activity in the mutant UTR (** represents $P < 0.01$). (E) siRNA-PDCD4 significantly decreased the protein level of PDCD4 in cells transfected with miR-497 inhibitor. (F) Knock down of PDCD4 blocked the increased cleaved PARP induced by miR-497 inhibitor under TMZ treatment.

ment in drug resistance [15]. It will be important to exploit the emerging knowledge of miRNAs in the development of new human therapeutic applications for overcoming anticancer drug resistance.

Previous reports show that miR-497 is downregulated in hepatocellular carcinomas and breast cancers, functioning as a tumor suppressive miRNA [10]. However, our experimental data, along with our analysis of a published dataset, demonstrate that miR-497 is overexpressed in glioma. Hypoxia is a common microenvironment for all kinds of cancer. Previous studies have shown that hypoxia induces miR-497 expression in retinoblastoma cells. We show that hypoxia can induce the expression of miR-497 in glioma cells. The PHD enzyme inhibitor DMOG also increases miR-497 expression in glioma cells, suggesting that HIF1α may regulate miR-497 expression. Chromatin immunoprecipitation assay confirmed that HIF1α binds the conserved binding sequences in the HRE1 (Fig. 3B) at the promoter of pri-miR-497, and thus regulates the expression of miR-497 at transcriptional level. We are the first to show how hypoxia induces the miR-497 expression in glioma cells.

Our data demonstrated that hypoxia-induced miR-497 enhanced glioma cell resistance to TMZ by targeting PDCD4. U251 cells and U87 cells overexpressing miR-497 were more resistant to TMZ treatment in vitro, as compared to the control cells. In contrast, the inhibition of miR-497 enhanced apoptosis and increased the sensitivity of glioma cells to TMZ treatment. Knock down of PDCD4 level in U251 cells transfected with miR-497 inhibitor could block the increased cleaved PARP induced by miR-497 inhibitor under TMZ treatment. The data indicate that PDCD4 is required for the role of miR-497 involved in drug resistance. There observations show that miR-497 might be a molecular target for TMZ sensitization in glioma.

In conclusion, our results demonstrate that miR-497 is significantly induced by hypoxia and an important regulator of TMZ resistance by targeting PDCD4. The finding that miR-497 is a predictor of response to TMZ in glioma cells underlies the importance of miR-497. Our results suggest that miR-497 represents an potential target for drug therapy via blocking miR-497 with antagomiRs in glioma tumors.

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