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ORIGINAL RESEARCH

Long noncoding RNA UBE2R2-AS1 promotes glioma cell apoptosis via targeting the miR-877-3p/TLR4 axis

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Introduction: Brain glioma is the most common type of primary malignancy in the central nervous system (CNS), with high recurrence and mortality rate, especially glioblastoma (GBM). Recent evidence suggests a role for many long noncoding RNAs (lncRNAs) in the pathogenesis, proliferation, apoptosis, metastasis, and chemotherapeutic resistance of cancer cells. Although the functions of some lncRNAs in the occurrence and development of gliomas have been confirmed, detailed mechanisms of action are lacking. Furthermore, the biological roles of many other lncRNAs in glioma have not been reported at all.

Methods: In this study, we identified a novel lncRNA, UBE2R2-AS1, which was dramatically downregulated in glioma compared with normal tissue, by performing microarray detection of six pairs of glioma samples and adjacent normal tissues. In vitro experiments demonstrated that UBE2R2-AS1 regulated glioma cell proliferation, apoptosis, and migration.

Results: UBE2R2-AS1 acted as a competing endogenous RNA (ceRNA) to target Toll-like receptor 4 (TLR4) mRNA by binding to miR-877-3p. Furthermore, lncRNA UBE2R2-AS1 suppressed glioblastoma cell growth, migration, and invasion, as well as promoting cell apoptosis by targeting miR-877-3p/TLR4 directly.

Conclusion: This information regarding UBE2R2-AS1 and its glioma-related molecular mechanisms will aid the future identification of new lncRNA-directed diagnostics and drug-targeting therapies.

Keywords: apoptosis, brain glioma, lncRNA, miR-877-3p/TLR4, UBE2R2-AS1

Introduction

Brain glioma is the most common type of primary malignancy in the central nervous system (CNS), with high recurrence and mortality rate, especially glioblastoma (GBM, WHO grade IV).[1] Globally, 11 million people are diagnosed with cancer each year, and this number is projected to reach 16 million in 2020.[2,3] About 200,000 patients are newly diagnosed with malignant glioma every year, and this number is increasing annually.[4] Despite advances in multiple modalities of glioma therapy including surgical resection, radiotherapy, and chemotherapy,[5] the overall survival rate for glioma patients remains low, especially in the case of glioblastoma, which has a median survival rate of 14.6 months.[6] The classification of tumors of the CNS in 2016 represented the first diagnostic modality of CNS tumors in the molecular field. This classification redefined some tumors or subtypes based on molecular gene characteristics and further emphasized the role of genes in the diagnosis and treatment of CNS tumors.[7] However, the therapeutic effects for glioma are limited because its molecular pathogenesis remains

unclear. The specific molecular mechanism of the occurrence and development of glioma is yet to be elucidated, which causes great difficulties in the diagnosis and treatment of human brain glioma.

Only 1–2% of the entire human genome encodes proteins, the rest consisting of non-coding sequences.[8] The complexity of the human genome transcriptome has transformed our traditional understanding of the potential of RNA, and has spurred research on the important roles of long noncoding RNA (lncRNAs) in transcription.[9,10] LncRNAs are non-protein-coding transcripts and range in length from nearly 200 to more than 100000 nucleotides.[11] Previously, lncRNAs have been considered to be transcriptional biological noise.[12] However, recent studies suggest that lncRNAs are not only involved in multiple normal biological processes, but also in the occurrence and progression of several cancers, including glioma.[13,14] For example, lncRNA H19, a proto-oncogene, has been shown to induce upregulation of miRNA-675 expression to promote glioma cell invasion.[15] Furthermore, lncRNA NEAT1 promotes the occurrence of glioma by regulating miRNA-449b-5p.[16] Wang et al found that an anti-oncogenic lncRNA, ENST00462717, may suppress the occurrence and development of glioma through the MAPK pathway.[17] LncRNA MALAT1 increases the sensitivity of glioma cells to temozolomide and downregulates the protein expression of ZEB1.[18] As there is a body of evidence supporting several important roles for lncRNAs in the biology of human glioma, they have become promising targets for diagnosis and potential therapy.

In this study, we identified a new lncRNA, UBE2R2-AS1, as a potential diagnostic biomarker and therapeutic target for glioma. We performed microarray detection in six pairs of glioma samples and adjacent normal tissues, and the results indicated that lncRNA UBE2R2-AS1 was dramatically downregulated in glioma. Therefore, we propose that lncRNA UBE2R2-AS1 may act as a tumor suppressor in glioma, and further demonstrated that UBE2R2-AS1 promoted apoptosis of glioma cells via regulating the miR-877-3p/Toll-like receptor 4 (TLR4) axis.

Material and methods
Patients and samples
From 2012 to 2017, 122 brain glioma samples were surgically resected at the Xinqiao Hospital of the Third Military Medical University (grade I–II in 37 cases and grade III–IV in 85 cases). As controls, 25 non-tumor brain samples were collected from craniocerebral decompression performed for traumatic brain injury. All samples were frozen in liquid nitrogen immediately after resection and stored at −80 °C until use. This study was approved by Xinqiao Hospital Ethical Committee and informed consent was obtained from all patients. All clinical pathological and biological data were available for those patients. Both tumor and non-tumor samples were confirmed by pathological examination. No patients received chemotherapy or radiotherapy prior to surgery. All participants provided written informed consent in accordance with the Declaration of Helsinki.

Cell culture
Human glioma cell lines U251, A-172, U87-MG, and U373 as well as normal cell line HA1800 were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS, HyClone), 100 U/ml penicillin, and 100 mg/ml streptomycin in humidified air at 37 °C with 5% CO_2.

Lncrna microarray analysis
The integrity of the final RNA preparation was assessed using an RNA nano-chip on an Agilent 2100 Bioanalyzer; RNA yield was determined by Nanodrop 2000 (Thermo Scientific) measurements. In total, 200 ng of purified RNA was used to generate cDNA for expression analysis on Affymetrix GeneChip® Human Transcriptome Array 2.0 arrays. Washing of arrays was performed using an Affymetrix GeneChip 450 Fluidics station. Scanning of arrays was performed with an Affymetrix 3000 GeneChip Scanner. Total RNA was isolated by conventional procedures (QIAGEN RNAeasy Mini Kit). Microarray data were normalized by robust multichip average in the oligo BioConductor package, which normalized the intensity values at the transcript level and collapsed probes into "core" transcripts based on annotations provided by Affymetrix. Heat maps were generated and hierarchical clustering were performed using GenePattern (Broad Institute) and MeV (MultiExperiment Viewer).

Construction of reporter and recombinant vectors
To construct expression vectors, the full-length sequence of UBE2R2-AS1 was amplified (the primer pairs used for this analysis are described in Additional S1). Polymerase

chain reaction (PCR) products were first sub-cloned into the pcDNA3.1 using BamhI/XhoI sites (Invitrogen) and subsequently cloned into the lentiviral plasmid pCDH (Addgene) by BamHI/NotI sites (Invitrogen). To construct luciferase reporter vectors, TLR4 3′-untranslated regions (UTRs) and UBE2R2-AS1 cDNA fragments containing predicted potential miR-877-3p binding sites or mutant sites were amplified by PCR, and then cloned into the psicheck2 Report Luciferase vector using NotI/XhoI sites (Invitrogen).

Lentivirus packaging
HEK293T cells were cultured in DMEM with 10% FBS and transfected with 3 µg plasmid pCDH-UBE2R2-AS1, 1 µg plasmid pMD2.G, and 3 µg plasmid psPAX2 using Lipofectamine 2000 reagent (Invitrogen). After incubation overnight, the medium was replaced with 10 ml fresh medium. The virus-containing supernatants were collected 48 and 72 h after transfection and filtered using a 0.45 µm cellulose acetate filter (Sigma-Aldrich).

Cell transfection
Recombinant lentiviruses containing full-length UBE2R2-AS1 and the control lentiviruses were obtained from Guangzhou Saiqing Biological Technology Company Ltd (Guangzhou, China). Transfection of glioma cell lines was performed by exposing them to dilutions of the viral supernatant in the presence of polybrene (5 mg/ml) for 72 h. Alterations of UBE2R2-AS1 in those cells were confirmed by real-time PCR before further analysis.

Quantitative Real-Time PCR
Total cellular RNA was isolated using an RNeasy Mini Kit (QIAGEN). cDNA was prepared using a cDNA Reverse Transcription Kit (Invitrogen, Thermo Fisher Scientific). Quantitative real-time PCR (qRT-PCR) was performed on a QS6 Fast Real-Time PCR system (Thermo Fisher Scientific) using either SYBR Green Master Mix or TaqMan Master Mix (Invitrogen, Thermo Fisher Scientific). Comparative quantification was performed using the $2^{-\Delta\Delta Ct}$ method. Primers can be found in Supplementary S1.

Luciferase reporter assay
Cells were co-transfected with either an empty vector or miR-877-3p and a luciferase reporter comprising 3′-UTRs of TLR4, wild-type, or mutant UBE2R2-AS1 fragments, using Lipofectamine 2000 (Invitrogen) in a 96-well format. Cells were harvested 48 h after transfection, and luciferase activity was measured by chemiluminescence in a luminometer (PerkinElmer Life Sciences) using the Dual-Luciferase reporter assay system (Promega).

RNA immunoprecipitation assay
RNA immunoprecipitation (RIP) was performed using a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore) and the Ago2 (Millipore) antibody based. Briefly, cells were lysed in RIP lysis buffer, then 100 µl of whole cell extract was incubated with RIP buffer containing A+G magnetic beads conjugated with human anti-Ago2 antibody, normal mouse IgG (Millipore) as a negative control, and anti-snRNP70 as a positive control (Millipore). Samples were incubated with proteinase K with shaking to digest the protein and immunoprecipitated RNA was isolated. qRT-PCR was performed to detect UBE2R2-AS1, TLR4 and miR-877-3p in the precipitates.

Cell Counting kit-8 (CCK8) assay
Cells at a concentration of 5×10^3 per well were seeded in a 96-well plate and incubated for 24, 48, and 72 h, respectively. Cell proliferation was measured using CCK8 assays (Beyotime). Absorbance (A) was then recorded at 450 nm using Elx800 Reader (Bio-Tek Instruments Inc.).

Flow-cytometric analysis of apoptosis
U373 and U87 cells were transiently transfected with the control lentiviruses, recombinant lentiviruses UBE2R2-AS1, or recombinant lentiviruses UBE2R2-AS1 with miR-877-3p mimics. Cells were harvested at 48 h post-transfection by trypsinization. Cell apoptosis was analyzed by staining cells with allophycocyanin (APC) annexin V and propidium iodide (PI) (both from BD Biosciences). Briefly, cells were suspended in 1×binding buffer at a concentration of 1×10^6 cells/ml. The cell suspension (100 µl) was then transferred to a 1.5-ml Eppendorf tube, mixed with 5 µl APC Annexin V and 10 µl PI, and incubated for 20 min at room temperature in the dark. Within 1 h, the samples were analyzed by flow cytometry.

Wound healing assay
U373 and U87MG cells were seeded in a six-well plate and cultured in the incubator to obtain 100% confluence before transfection. At the 0 h time point, the monolayer cell in each well was scraped by a sterile needle to generate a clear line; afterwards, cells were transfected with the control lentiviruses, recombinant lentiviruses UBE2R2-AS1, or recombinant lentiviruses UBE2R2-AS1 with miR-877-3p

mimics. Pictures were taken using a Nikon Eclipse TS100 microscope (Nikon, Japan) at the 0 h and 48 h post-injury time points.

Colony formation assay

The transfected U373 or U87 cells were placed in a fresh six-well plate and maintained in DMEM containing 10% FBS. After 24 h, a new medium containing selection agent G418 (400 mg/ml) was applied. After 14 days, cells were fixed with methanol and stained with 0.1% crystal violet. Visible colonies were manually counted.

Western blotting

The transfected U373 and U87 cells were lysed with denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer using standard methods. Protein lysates were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with Tris-buffered saline containing 0.1% Triton X-100 and 5% nonfat milk overnight at 4 °C, before being incubated with anti-human TLR4, MYD88, FADD, CASP8, CASP7, CASP3, Bcl2 (Proteintech), and anti-β-actin antibody (Abcam) at 4 °C overnight. After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-IgG at room temperature for 2 h. Signal detection was carried out with an electrochemiluminescence system (Apbiotech).

Statistical analysis

All experiments were performed at least three times. Data are presented as the mean ± standard error of mean (SEM). Analyses were conducted with the SPSS 23.0 software, using the unpaired Student's t-test for comparisons between two groups or one-way analysis of variance for multiple comparisons. $P<0.05$ was considered statistically significant.

Results
UBE2R2-AS1 is downregulated in glioma and correlated with patient clinical-pathological characteristics

To establish the expression profile of the key putative lncRNAs and genes in glioma patients and the paired tumor adjacent controls, we performed microarray analyses followed by hierarchical clustering, which revealed distinguishable expression patterns between tumors and non-tumor brain samples (Figure 1A). Using an absolute

fold change of at least 2.0 and an adjusted P-value of less than 0.05, we observed that the expression levels of four lncRNAs were significantly upregulated in the tumor region, while those of five 5 lncRNAs were downregulated compared with the control group. The subsequent Gene Ontology (GO) and pathway enrichment analyses predicted several biological processes, highlighting the apoptotic-related signature, including TLR and nuclear factor (NF)-κB networks (Figure 1B and C). The microarray data also showed that expression of lncRNA UBE2R2-AS1 was most significantly decreased in the human glioma region. This finding was confirmed via two approaches: technical validation using qRT-PCR on the aforementioned human tissues (tumor tissues vs non-tumor brain tissues; Figure 1D), and biological validation using glioma cell lines compared with the HA1800 cell line as a control (Figure 1E).

In order to determine the clinical significance of lncRNA UBE2R2-AS1 in glioma, we investigated the correlation of lncRNA UBE2R2-AS1 expression with human glioma clinicopathological characteristics (1). The data clearly showed that the lncRNA UBE2R2-AS1 expression level was significantly negatively correlated with tumor World Health Organization (WHO) grades and epilepsy, but had no significant associations with age, gender, tumor diameter, or Karnofsky performance score. Taken together, these results indicate that lncRNA UBE2R2-AS1 might play an important part in the regulation of glioma progression.

Overexpression of UBE2R2-AS1 inhibits proliferation and migration but induces apoptosis of glioma cells

Based on our microarray data and the subsequent validation analysis, we hypothesized that UBE2R2-AS1 might suppress the malignant progress of glioma by triggering cell apoptosis. To elucidate the role of UBE2R2-AS1 in glioma, we selected U87 and U373 as representatives from commonly used glioma cell lines, and transfected them with UBE2R2-AS1 or a control fragment. A significant increment in UBE2R2-AS1 expression could be detected in U87 and U373 cells when transfected with UBE2R2-AS1 ($P<0.01$, Figure 2A), which markedly suppressed the proliferation of these two types of glioma cells ($P<0.01$, Figure 2B). Furthermore, the colony formation assay indicated that cell survival was significantly restrained upon overexpression of UBE2R2-AS1 ($P<0.01$, Figure 2C), in line with the observation of proliferation inhibition. Next, we asked whether UBE2R2-AS1
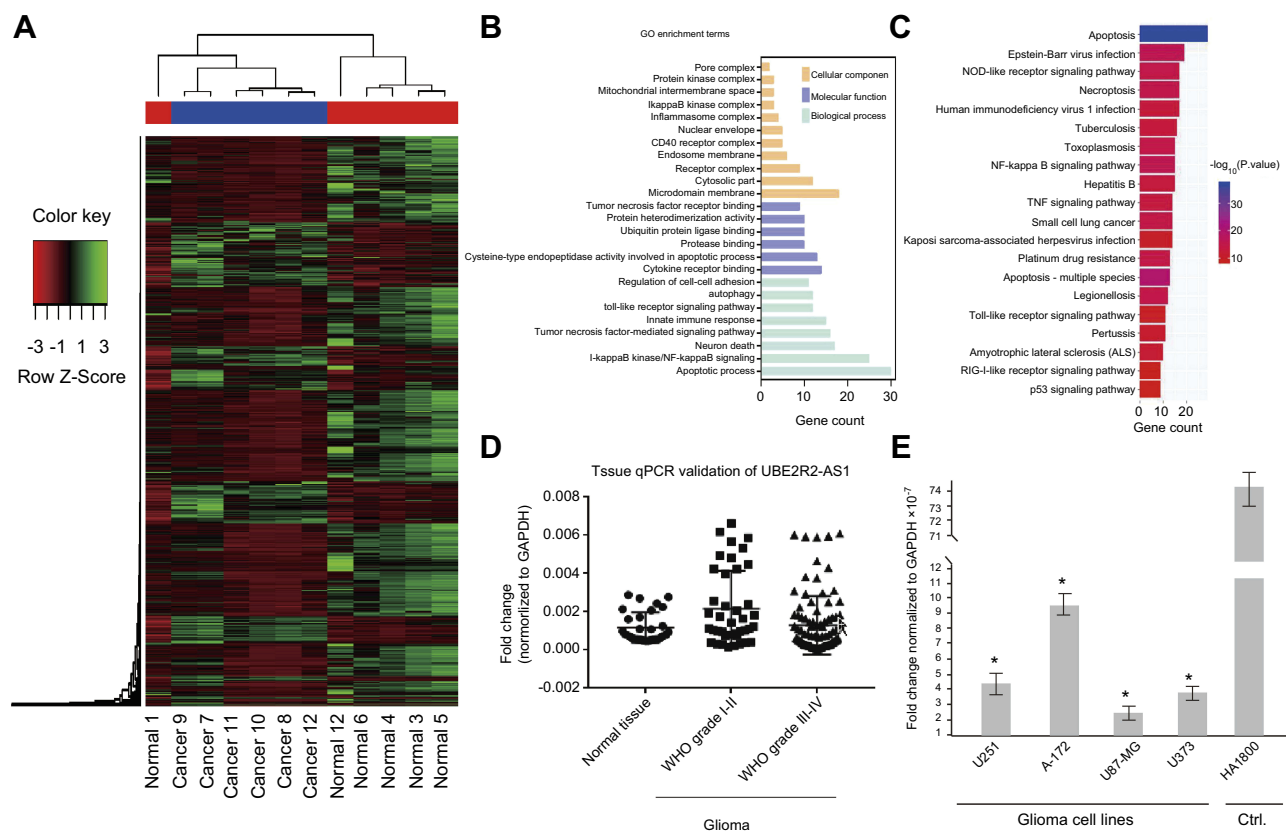
Figure 1 Aberrantly expressed lncRNAs by microarray-based detection and correlation analysis between UBE2R2-AS1 expression and clinicopathologic characteristics of glioma patients. (**A**) Microarray heatmap display the typical lncRNAs and genes set expression classification and relations between the tumor and adjacent tissue from six typical patients. (**B**) Gene Ontology enrichment analysis genes co-expression with lncRNA UBE2R2-AS1. (**C**) KEGG enrichment analysis genes co-expression with lncRNA UBE2R2-AS1. (**D**) 122 tissue qRT-PCR validation assay proves that UBE2R2-AS1 are downregulated in glioma, non-tumor brain tissues is the normal control. (*$P<0.05$, **$P<0.01$).(**E**) qRT-PCR validation assay proves that UBE2R2-AS1 are differently expressed in 4 glioma cell lines, HA1800 is the control cell line. (*$P<0.05$, **$P<0.01$).

had an effect on the regulation of cell apoptosis. Indeed, forced expression of UBE2R2-AS1 resulted in a significantly higher proportion of Annexin V-positive but propidium iodide-negative cells, indicating early apoptotic events in both U87 and U373 cells (Figure 2D). In addition, wound healing assays showed that overexpression of UBE2R2-AS1 substantially slowed down the gap closure, that is, the migration of U87 and U373 cells (Figure 2E). Collectively, these results suggest that UBE2R2-AS1 could suppress cell growth and induce apoptosis in glioma.

UBE2R2-AS1 and miR-877-3p inversely regulate cell apoptosis by targeting TLR signaling pathway in glioma

Given the high likelihood of UBE2R2-AS1 having a role in apoptosis regulation, based on the GO data (Figure 1B and C) and aforementioned in vitro assays (Figure 2), we sought to determine the relevant signaling pathways associated with UBE2R2-AS1. Hence, we assessed the expression levels and

phosphorylation levels of several key proteins in apoptosis-associated signaling pathways, including mitogen-activated protein kinases (MAPKs) and NF-κB signals. The functional proteins of MAPKs signaling pathway, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38-MAPK, have pivotal roles in cell proliferation and tumorigenesis. In U87 cells, forced expression of UBE2R2-AS1 resulted in markedly higher phosphorylation levels of ERK1/2, but not of p38 and phospho-JNK (Figure 3A), nor IκBα and phospho-IκBα (Figure 3B). Further, UBE2R2-AS1 upregulation significantly increased the protein expression levels of Caspase-8, Caspase-3, and Caspase-7, but inhibited the protein expression level of BCL2 in glioma cells (Figure 3C). These results support the postulation that UBE2R2-AS1 is involved in the apoptotic signaling pathway.

Since our aforementioned microarray and bioinformatic analysis indicated a functional association of UBE2R2-AS1 with the TLR signaling pathway, classical genes in the pertinent network were selected for validation, and their RNA expression in UBE2R2-AS1-overexpressing glioma cells or

Table 1 Correlations between lncRNA UBE2R2-AS1 expression and clinicopathological characteristics of glioma patients

Characteristic	Number	lncRNA UBE2R2-AS1 expression		P-value
		Low	High	
Gender				0.099
Female	52	31	21	
Male	70	30	40	
Age				0.825
<60 years	96	47	49	
≥60 years	26	14	12	
Tumor diameter				0.360
<5cm	70	38	32	
≥5cm	52	23	29	
WHO				0.018*
I–II	37	12	25	
III–IV	85	49	36	
KPS[a]				0.365
<80	58	32	26	
≥80	64	29	35	
Epilepsy				0.029*
No	86	49	37	
Yes	36	12	24	

Notes: [a] KPS, karnofsky performance score.
* The values had statistically significant differences ($p < 0.05$).

controls was determined by qRT-PCR. The results indicated that UBE2R2-AS1 promoted the expression of TLR4, FADD, MYD88, CASP3, CASP8, and CASP9 (Figure 3D), in line with the western blot data. Of note, our bioinformatics analysis suggested that TLR4 and CASP8 may be target molecules of miR-877-3p and miR-7641, respectively. Intriguingly, UBE2R2-AS1 negatively regulated the expression of miR-877-3p and miR-7641 (Figure 3D).

To elucidate the regulatory network of lncRNA/miRNA/target molecules, we first examined the expression levels of caspase-8 and TLR4 upon forced expression of miR-877-3p mimics and miR-7641 mimics in glioma cells, respectively. Interestingly, both protein and gene expression results showed that miR-877-3p significantly reduced the expression of caspase-8 and TLR4, whereas miR-7641 had no such effects (Figure 3E and F). Next, we asked whether the above two miRNAs could control the expression of UBE2R2-AS1, and found that only miR-877-3p, but not miR-7641, negatively regulated UBE2R2-AS1 (Figure 3E); this suggested that miR-877-3p/UBE2R2-AS1 may inversely modulate apoptosis. To

consolidate the hypothesis, either UBE2R2-AS1 alone or UBE2R2-AS1 alongside miR-877-3p mimics was transfected into U87 cells, and the post-transcriptional expression levels of apoptotic readout genes FADD, MYD88, and Caspase-8 were analyzed by immunoblotting. The results demonstrated that the apoptosis-promoting effect of UBE2R2-AS1 could be antagonized by miR-877-3p (Figure 3G). Collectively, our data indicate that the UBE2R2-AS1/miR-877-3p/TLR4/apoptotic signaling pathway axis may be involved in the regulation of glioma progression.

UBE2R2-AS1 is a target of miR-877-3p and regulates TLR4 expression via acting as a "molecular sponge" for miR-877-3p

To further explore the molecular mechanisms of UBE2R2-AS1, has-miR-877-3p, and TLR4, we proposed miR-877-3p binding sites on the UBE2R2-AS1 and TLR4 3′-UTRs as per bioinformatic predictions (Figure 4A; LncBase Predicted v.2 and TargetScan). To verify the binding between UBE2R2-AS1 and has-miR-877-3p, and between TLR4 and has-miR-877-3p, the wild-type vectors of UBE2R2-AS1 and TLR4 in the psiCHECK2 vector series were constructed, respectively. For the mutant vector, the specific binding site sequence and the mutant sequence are shown in Figure 4A. U87 cells were co-transfected with wild-type and mutant vectors of UBE2R2-AS1 and TLR4, alongside miR-877-3p mimics and negative control (NC) mimics, respectively. After 48 h, the relative luciferase levels of the groups were determined. The luciferase assay showed that has-miR-877-3p significantly inhibited the luciferase activity of the reporter genes psiCHECK2-UBE2R2-AS1 (~75% inhibition, Figure 4B) and psiCHECK2-TLR4 3'UTR (~71.6% inhibition, Figure 4C). These results demonstrate that has-miR-877-3p binds to UBE2R2-AS1 as well as to TLR4 3′-UTR.

AGO2 protein is a core component of the miRNA synthesis RISC complex. To further demonstrate the binding between has-miR-877-3p and UBE2R2-AS1 or TLR4, RIP of AGO2 was performed in U87 cells. The RIP experimental product was detected by qRT-PCR; the results confirmed that has-miR-877-3p not only binds to UBE2R2-AS1 but also to TLR4 (Figure 4E).

To validate the possible competing endogenous RNA (ceRNA) mechanisms of UBE2R2-AS1, has-miR-877-3p, and TLR4, an overexpression vector for pcDNA3.1-UBE2R2-AS1 was constructed. Luciferase assays were performed in the experimental groups shown in Figure 4D. The results showed that overexpression of UBE2R2-AS1
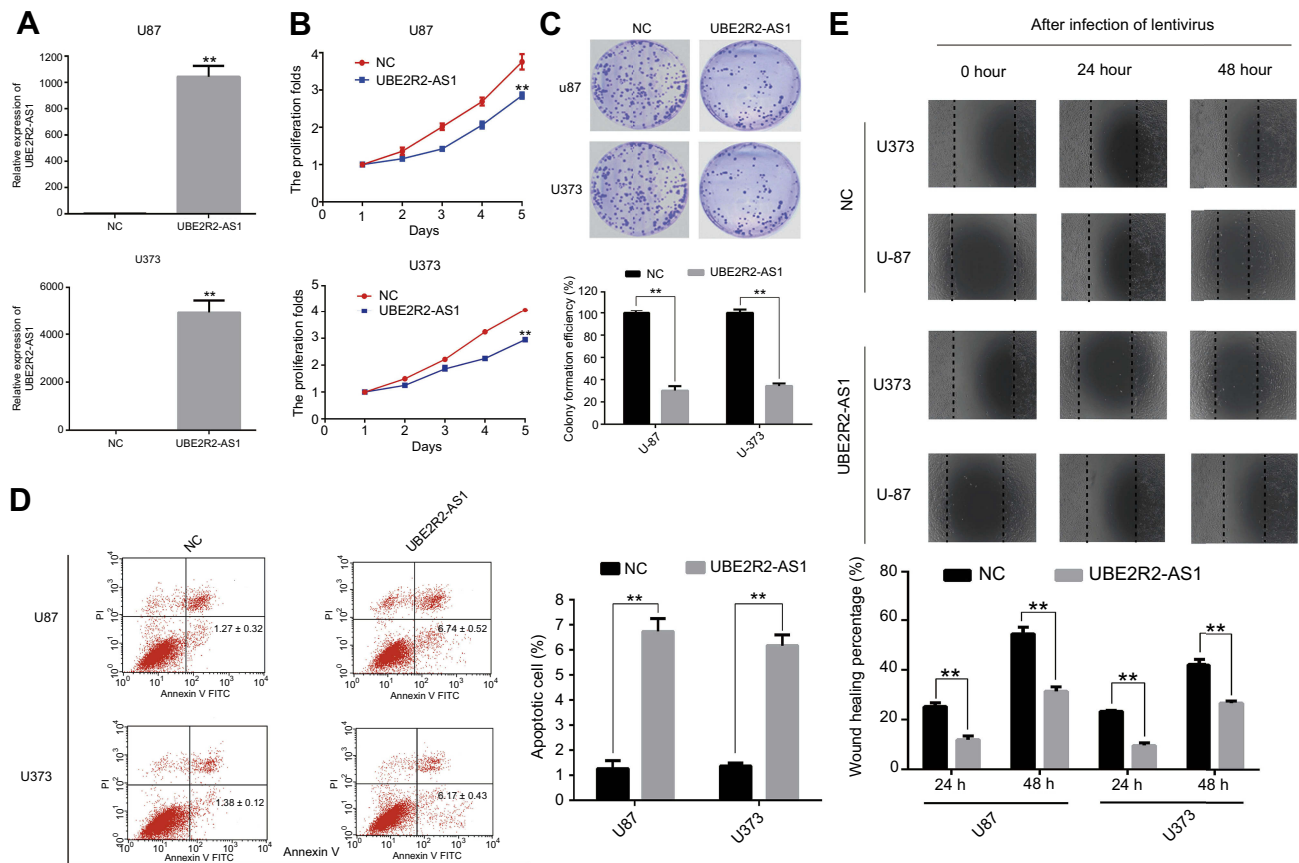
Figure 2 Overexpression of UBE2R2-AS1 inhibited proliferation and migration while inducing cell apoptosis in glioma cells. (**A**) Relative expression of UBE2R2-AS1 in U87 and U373 cells was detected through qRT-RCR. (**B**) CCK8 assay was employed to examine proliferation rates of U87 and U373 cells. (**C**) Cell proliferation was detected by clone formation assay in U87 and U373 cells transfected with UBE2R2-AS1 and NC. (**D**) Flow cytometry was performed to detect the effects of UBE2R2-AS1 on apoptosis in U87 and U373 cells. (**E**) U87 and U373 cells were transfected with UBE2R2-AS1 or NC respectively, and in vitro migration was assessed by wound healing assay. (*$P<0.05$, **$P<0.01$).

significantly upregulated TLR4 transcription in U87 cells, whereas has-miR-877-3p mimics significantly inhibited this effect, and even reduced TLR expression. These results also suggest that UBE2R2-AS1 might function as an internal sponge for has-miR-877-3p by positively regulating the expression of TLR4. In addition, the results of UBE2R2-AS1 overexpression and miR-877-3p mimics further confirmed that UBE2R2-AS1 sponges miR-877-3p by indirectly modulating TLR4 expression (Figure 4F). To summarize, UBE2R2-AS1 acts as a ceRNA to target TLR4 mRNA by binding miR-877-3p, thus leveraging the expression of TLR4 and imposing an additional level of post-transcriptional regulation.

UBE2R2-AS1 regulates glioma cell proliferation, apoptosis, and migration via miR-877-3p inhibition

To further determine whether UBE2R2-AS1 regulated the proliferation, apoptosis and migration of glioma cells via inhibition of miR-877-3p, we performed CCK8, clone formation, fluorescence-activated cell sorting (FACS), and wound healing assays. The CCK8 assay was employed to examine the proliferation rates of UBE2R2-AS1-overexpressing glioma cell lines treated with or without miRNA-877 mimics. The growth curves indicated that overexpression of UBE2R2-AS1 substantially suppressed the proliferation of U87 and U373 cells, whereas miR-877-3p restrained the proliferative capacity of glioma cells (Figure 5A).

Clone formation assays were performed using UBE2R2-AS1 or UBE2R2-AS1 with miRNA 877 mimics transfected into U87 and U373 cells. Colony formation efficiency was observed in U87 and U373 cells treated with UBE2R2-AS1, miRNA 877 mimics, or NC by clone formation analysis, consistent with the CCK8 assay (Figure 5B).

Flow cytometry was performed to determine the percentage of apoptotic cells among the U87 and U373 cells. The FACS results showed that overexpression of UBE2R2-AS1 induced cell apoptosis, whereas simultaneous overexpression of miR-877-3p reversed this performance (Figure 5C).
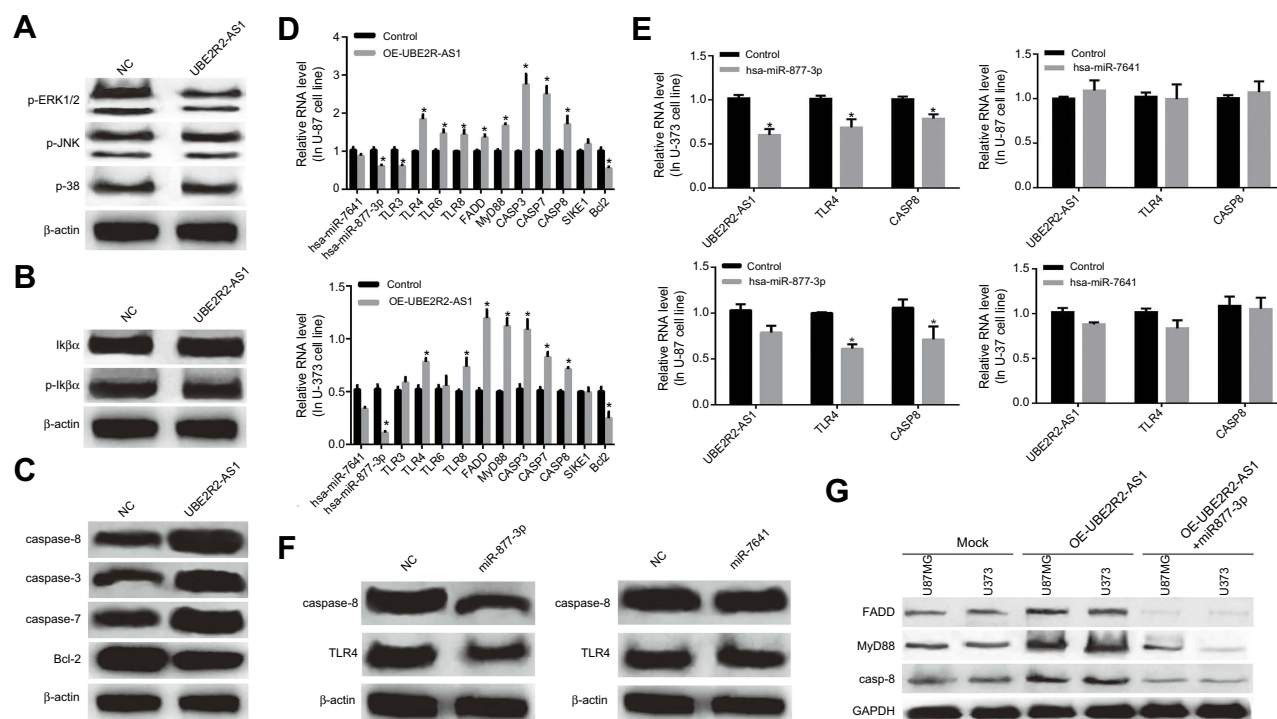
Figure 3 UBE2R2-AS1 and miR-877-3p inversely regulated cell apoptosis by Toll-like receptor signaling pathway in glioma.(**A**) Western blot analysis of *p*-ERK2/1, *p*-JNK, *p*-38 protein level in UBE2R2-AS1 overexpressed U87 cells, with β-actin as an endogenous reference.(**B**)Western blot analysis on IκBα and *p*-IκBα in UBE2R2-AS1 overexpressed U87 cells, with β-actin as an endogenous reference.(**C**)The expression level of apoptosis-related proteins was detected in UBE2R2-AS1 transfected U87 cells through western blotting. β-actin was used as an endogenous reference.(**D**) The expression of TLR-related genes and apoptosis-related genes was analyzed by qRT-PCR assay and GAPDH was used as an internal control. (*$P<0.05$.) (**E**) Relative RNA level of caspase-8 and TLR4 was detected in miR-877-3p or miR-7641 mimics transfected U87 and U373 cells through qRT-PCR assay. GAPDH was used as an internal control. (**F**)Western blot analysis on caspase-8 and TLR4 in U87 cells transfected miR-877-3p or miR-7641 mimics, with β-actin as an endogenous reference. (**G**)Western blot analysis of FADD, MyD88 and caspase-8 protein level following treatment of U87 cells and U373 cells with pcDNA3.1-UBE2R2-AS1 and miR-877-3p mimics. β-actin was used as control.

In vitro migration was assessed by wound healing assays; U87 and U373 cells were transfected with UBE2R2-AS1 and UBE2R2-AS1 with miR-877 mimics, respectively. The results indicated that upregulation of UBE2R2-AS1 in glioma cells significantly suppressed cell migration, whereas inhibition of UBE2R2-AS1 by miR-877-3p mimics dramatically rescued the migration capacity of glioma cells (Figure 5D).

To summarize, our results indicate that UBE2R2-AS1 regulates glioma cell proliferation, apoptosis, and migration via inhibiting miR-877-3p. A model is shown Figure 6 to illustrate the mechanism of action.

Discussion

Infiltration of tumor cells into normal brain tissue makes complete surgical resection challenging and is the main cause of poor prognosis in patients with glioma.[19] In recent decades, advances in glioma research have mainly focused on tumor cell genomics and heterogeneity; this substantially facilitated the latest WHO 2016 glioma molecular classification on the one hand, but resulted in highly chemotherapy- and radiotherapy-resistant glioma cells on the other hand.[20]

However, temozolomide remains the only frontline therapeutic treatment for gliomas, resulting in an extraordinary drive to identify new biological mechanisms and concomitant drug targets. In the past few years, the involvement of lncRNAs has been reported in many cancer processes. Many lncRNAs can inhibit the occurrence and progression of gliomas by downregulating or activating key genes. These lncRNAs may be useful for potential therapeutic targets for glioma in the future.[15–18] For example, lncRNA HSP90AA1-IT1 was shown to be significantly upregulated in glioma tissues compared with para-carcinomic tissues using gene expression microarrays.[21] Similarly, lncRNA ENST00462717 was the most highly downregulated lncRNA in the microarray data, and was found to partially regulate the MDM2/MAPK pathway in glioma cells.[17] Yet, until now, the specific roles of most lncRNAs in glioma have not been elucidated.

In our research, we used microarray analysis to establish key pre-indicator lncRNA and gene expression profiles in six glioma patients and matched tumor-adjacent controls. Then, we identified the most significantly differently expressed lncRNA as UBE2R2-AS1, and validated this result using
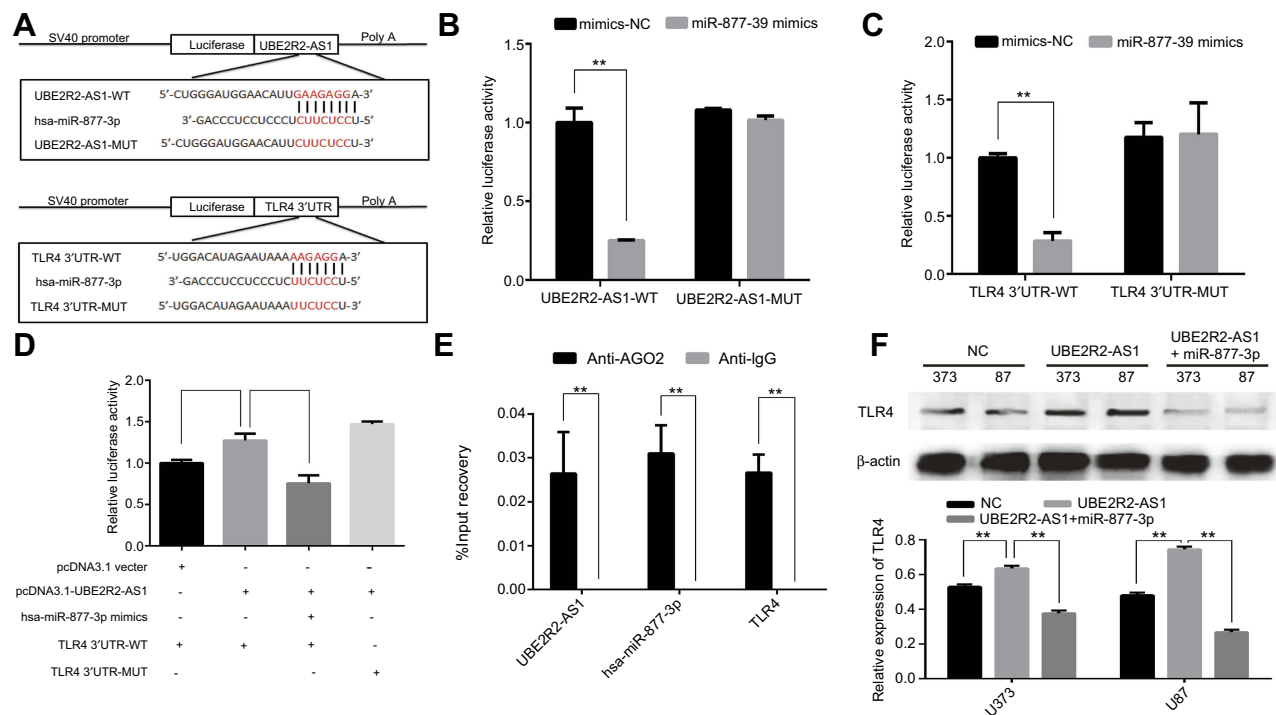
Figure 4 UBE2R2-AS1 is a target of miR-877-3p and regulates the expression of TLR4 as a molecular sponge for miR-877-3p. (**A**). Bioinformatics predicted miR-877-3p binding sites in UBE2R2-AS1 and TLR4 3′UTR. Mutation was generated on the UBE2R2-AS1 and TLR4 3′UTR sequence in the complementary site for the seed region of miR-877-3p. UBE2R2-AS1 containing wild type or mutant miR-877-3p binding sequence was cloned into psiCHECK-2 luciferase reporter. (**B**) Luciferase activity was measured in U87 cells co-transfected with wild type (UBE2R2-AS1-WT) or mutant (UBE2R2-AS1-MUT) UBE2R2-AS1 and miR-877-3p using the luciferase reporter assay. (**C**) Luciferase activity was measured in U87 cells co-transfected with wild type (TLR4 3′UTR -WT) or mutant (TLR4 3′UTR -MUT) TLR4 3′UTR and miR-877-3p using the luciferase reporter assay. (**D**) TLR4 3′UTR -WT or TLR4 3′UTR -MUT and miR-877-3p constructs were co-transfected into U87 cells cells with plasmids expressing UBE2R2-AS1 (pcDNA-3.1-UBE2R2-AS1) or with a control vector to verify the ceRNA activity of UBE2R2-AS1. Luciferase activity was measured in U87 cells 48 h after transfection. (**E**) RIP with mouse monoclonal anti-Ago2, preimmune IgG or input from U87 cells extracts. RNA levels in immunoprecipitates were determined by qRT-PCR. Levels of UBE2R2-AS1, miR-877-3p and TLR4 RNA were presented as percentages input recovery in Ago2 relative to IgG immunoprecipitates. (**F**) Western blot analysis of TLR4 protein level following treatment of U87 cells and U373 cells with pcDNA3.1-UBE2R2-AS1 and miR-877-3p mimics. β-actin was used as control.

122 glioma samples and 25 normal brain parenchymal samples. We characterized this further via cross-referencing for GO enrichment, which confirmed that UBE2R2-AS1 led to cell apoptosis as the most significant functional impact on glioma. Our data represent the first report of the function of UBE2R2-AS1 in glioma and its possible molecular biological mechanisms. More recently, the interaction between lncRNA and miRNA in the pathogenesis of cancer has received more attention. A large number of reports have indicated that lncRNAs exert their functions through sponging off miRNAs, serving as ceRNAs to compete with miRNAs that bind to mRNAs for degradation.[22,23] Previous data have shown that miR-21 expression decreased significantly after overexpression of the glioma tumor suppressor gene lncRNA CASC2, and there was a mutual inhibition between Argonaute 2-mediated CASC2 and miR-21.[24] Blocking CASC2 by upregulation of miRNA-152 prevents tumor stem cell growth in human glioblastoma.[25] Li et al[26] found that miR-877-3p suppressed the proliferation and tumorigenesis of bladder cancer through arrest of the G1 phase of the cell cycle and confirmed

that the correlation between p16 activation and miR-877-3p was due to direct binding. Based on bioinformatic analysis, we predicted that UBE2R2-AS1 contains a binding site with has-miR-877-3p, using the LncBase Predicted v.2 website. We discovered a binding site of has-miR-877-3p from TLR4 3′-UTR sequences using the TargetScan website. To verify the binding between UBE2R2-AS1 and has-miR-877-3p and between TLR4 and has-miR-877-3p, we constructed wild-type vectors of UBE2R2-AS1 and TLR4 in the psiCHECK2 vector series. The results further suggested that has-miR-877-3p could target both UBE2R2-AS1 and TLR4.

TLR4, the most widely studied member of the TLR family, was confirmed as the long-sought receptor that responds to bacterial lipopolysaccharide (LPS).[27] TLR4 elicits innate and adaptive immune responses via MYD88- and TRIF-dependent signaling pathways, leading to the production of pro-inflammatory cytokines or IFN-β.[28] TLR4 is reported to be expressed in the CNS, including in microglia, neurons, astrocytes, and neural stem cells.[29–32] TLR4 activation induces neuronal apoptosis via a TRIF-dependent signaling pathway;[33]
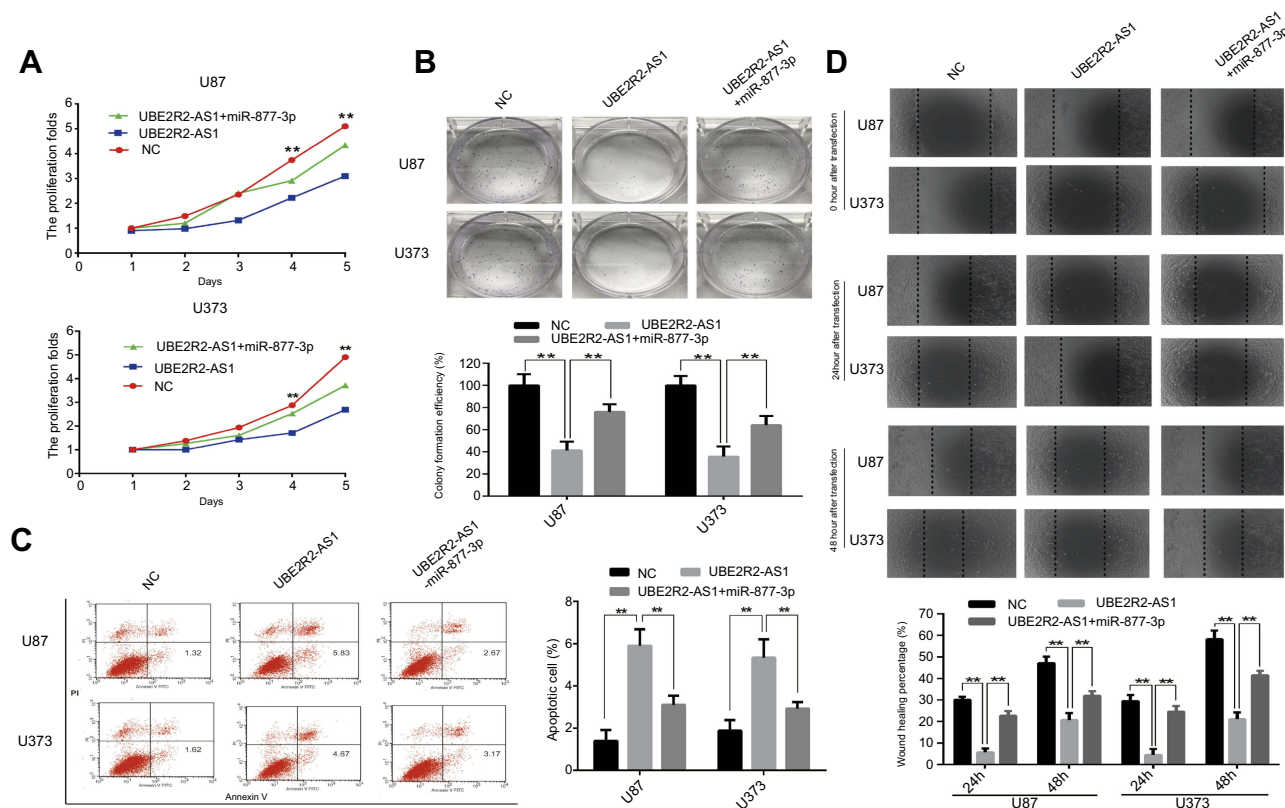
Figure 5 UBE2R2-AS1 regulated glioma cell proliferation, apoptosis and migration via miR-877-3p inhibition. (**A**) CCK8 assay was employed to examine proliferation rates in UBE2R2-AS1 overexpressed glioma cell lines with or without treatment of miRNA 877 mimics. (**B**) Clone formation assay were performed to determine the proliferation of UBE2R2-AS1 or UBE2R2-AS1 with miRNA 877 mimics transfected U87 and U373 cells. (**C**) Flow cytometry was performed to determine the percentages of apoptosis in U87 and U373 cells. (**D**) U87 and U373 cells were transfected with UBE2R2-AS1, UBE2R2-AS1 with miRNA 877 mimics or NC respectively, and in vitro migration was assessed by wound healing assay. (*$P<0.05$, **$P<0.01$).

however, its role in gliomagenesis is ambiguous. On the one hand, Hu et al[34] suggested that TLR4 reverses tumor differentiation in human glioma U251 cells that and it could be a biological target for glioma prevention and therapy. On the other hand, TLR4 is significantly decreased in cancer stem cells (CSCs) and suppresses CSC properties by reducing levels of retinoblastoma binding protein 5.[35] Nonetheless, both caspase 8- and 9-dependent cell death in human monocytic THP-1 cells occur when TLR4 is activated by LPS induction, suggesting that TLR4 may regulate the immune homeostasis via apoptotic mechanisms.[36] We found that overexpression of UBE2R2-AS1 significantly increased the protein expression levels of caspase-8, caspase-3, and caspase-7 in glioma cells, while reducing that of Bcl2. These results indicated that UBE2R2-AS1 was likely to be related to the apoptotic signaling pathway. To verify the possible ceRNA mechanisms involving UBE2R2-AS1, has-miR-877-3p, and TLR4, we constructed an overexpression vector for pcDNA3.1-UBE2R2-AS1. Our findings indicated that by binding miR-877-3p, UBE2R2-AS1 acts as a ceRNA of

miR-877-3p for the target TLR4 mRNA, thereby fine-tuning the expression of TLR4 via imposing an additional level of post-transcriptional regulation. When preparing our manuscript, a meta-analysis performed by Zhou et al[37] on datasets from The Cancer Genome Atlas reported a six-lncRNA signature for GBM prognosis, which indicated that UBE2R2-AS1 may be protective for glioma. Our findings from tumor tissue and cell-based assays in this study were in line with the above *in silico* study.

Conclusions

Our study provides the first data showing that lncRNA UBE2R2-AS1 suppresses glioma cell growth, migration, and invasion, as well as promoting glioma cell apoptosis by targeting miR-877-3p/TLR4 directly. Although the molecular mechanism underlying this regulatory effect needs further elucidation, our current findings focusing on the functions of lncRNA UBE2R2-AS1 and its glioma-related molecular mechanisms (Figure 6) will aid
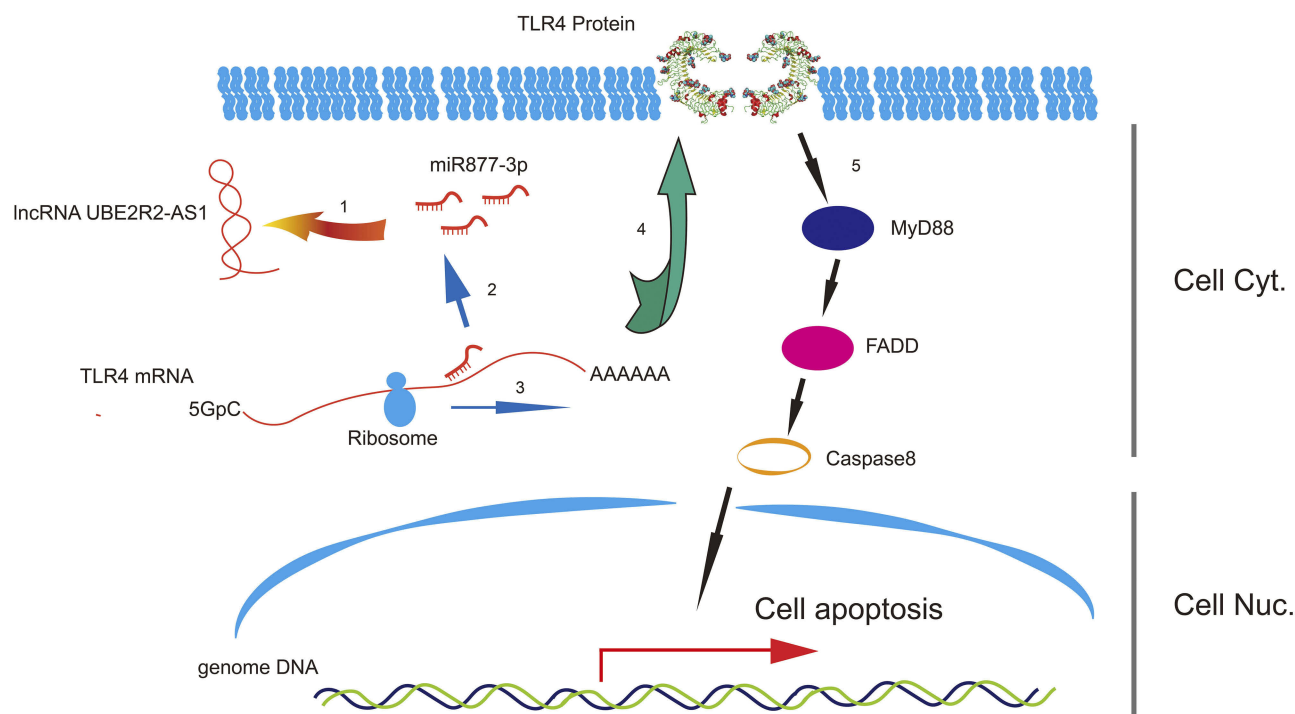
Figure 6 Molecular mechanism of the lncRNA UBE2R2-AS1.Step1 UBE2R2-AS1 takes sponging effect on miR-877-3p.
Step2 miR-877-3p losses interaction with TLR4 mRNA 3′UTR region.
Step3 TLR4 start translation for more protein for losing miR-877-3p control.
Step4 Upregulated TLR4 protein locates on the cell membrane and more sensitive to signal of environment.
Step5 TLR4 can initiate the cell apoptosis pathway via MyD88/FADD/Caspase8 Axis.

the identification of new lncRNA-directed diagnostics and drug-targeting therapy in the future.

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

Table S1 Primes for miRNAs, UBE2R2-AS1 and mRNAs in this study

Name	F & R Primes	Prime sequences from 5'-3'
miRNA prime with A tail	Reverse Transcription	CTCTACAGCTATATTGCCAGCCACACTAATTTTTTTTTTTTTTTT
hsa-miR-877-3p	F	TCCTCTTCTCCCTCCTCCC
	R	CTCTACAGCTATATTGCCAGCC
hsa-mir-7641	F	GTTTGATCTCGGAAGCTAAGCAG
	R	CTCTACAGCTATATTGCCAGCC
UBE2R2-AS1	F	GTCTGGGTAGTCAGCTGTGAGG
	R	TCTCCAGAGGCAGTGTTCCTC
TLR3	F	ACTACCTTTGCAACACTCCACCT
	R	TCAACAGGATACTGGTATTGATCATG
TLR6	F	GCATCATGGAACTGTGCAATG
	R	TGATCCTGCCAGCTGCTATG
TLR4	F	TATGATCCAGCAATCTCACTTCTGT
	R	ACAGTGATTGTGAAGAGTGCCAC
TLR8	F	TGCAATGTAGGTGTTCACCAGAG
	R	GCTCGCATGGCTTACATGAG
SIKE1	F	GGTGAGGAAGCAGTTTTGTTACC
	R	ATGAGTGAACCCAGTTGACCAC
CASP8	F	CCTGCTGAAGATAATCAACGACTATG
	R	AACTTTGTCCAAAGTCTGTGATTCAC
MyD88	F	GCCTGTCTCTGTTCTTGAACG
	R	CGTCCAGCAGCCTGCC
FADD	F	GGAGAAGGCTGGCTCGTC
	R	CTGTTGCGTTCTCCTTCTCTGTG
CASP7	F	ACGATGGCAGATGATCAGG
	R	CTTGATGGATCGCATGGTGAC
CASP3	F	GAGACAGACAGTGGTGTTGATGATG
	R	GACTGGATGAACCAGGAGCC
Bcl2	F	CCTGCATCTCATGCCAAGG
	R	CCAGAGAAAGAAGAGGAGTTATAATCC
U1(control)	F	GGGAGATACCATGATCACGAAGGT
	R	CCACAAATTATGCAGTCGAGTTTCCC
GAPDH(control)	F	GGAGCGAGATCCCTCCAAAAT

(Continued)

Table S1 (Continued).

Name	F & R Primes	Prime sequences from 5'-3'
	R	GGCTGTTGTCATACTTCTCATGG
UBE2R2-AS1-WT	BstBI-F	AAAATTCGAAgttaaccacttgagcaggtttca
	NotI-R	AAAAGCGGCCGCtggtgttcagtaatggcagatg
XW-UBE2R2-AS1-mut	BstBI-F	AAAATTCGAAgttaaccacttgagcaggtttca
	2R	GAAGAGGACCGTGGAACGAAACTATTATTGTCTCCTTCTC
	2F	CTGGGATGGAACATTGAAGAGGAACACTGCCTCTGGAGAGG
	NotI-R	AAAAGCGGCCGCtggtgttcagtaatggcagatg
TLR4-WT	pmel-F	AAAGTTTAAACtgagcaagtaacagaaagacaaatactg
	NotI-R	AAAAGCGGCCGCattttgagagagagaaagaaagagatcac
TLR4-mut	pmel-F	AAAGTTTAAACtgagcaagtaacagaaagacaaatactg
	2R	TGCAAAGAGGCCACCCCGTttaagtgcataatacagtattgttcattataC
	2F	GtataatgaacaatactgtattatgcacttaaACGGGGTGGCCTCTTTGCA
	NotI-R	AAAAGCGGCCGCattttgagagagagaaagaaagagatcac