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NCK1-AS1 Increases Drug Resistance of Glioma Cells to Temozolomide by Modulating miR-137/*TRIM24*

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Abstract

Background: Glioma is a common malignant tumor. The purpose of this study was to investigate the effect and molecular mechanism of long noncoding RNA (lncRNA) NCK1-AS1 on the drug resistance of temozolomide (TMZ) in glioma cells.
Methods: The fresh and recurrent glioma tissues and peritumoral brain edema (PTBE) were collected from the same patient. U251 and A172 cells were treated with TMZ to screen TMZ-resistant cells. The expression levels of NCK1-AS1, miR-137, or TRIM24 were detected by quantitative real-time polymerase chain reaction (qRT-PCR), Western blotting, *in situ* hybridization (ISH), or RNA pull-down assay. Cell viability was measured by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazoliumbromide (MTT) assay. In addition, the relationship between NCK1-AS1 and miR-137 or *TRIM24* and miR-137 was confirmed by dual luciferase activity assay.
Results: NCK1-AS1 expression was increased in regular and recurrent glioma tissues and TMZ-resistant cells. Cell viability was increased in TMZ-resistant cells, and the IC50 of TMZ also increased in TMZ resistant cells. However, knockdown of NCK1-AS1 inhibited these increases. Moreover, suppression of NCK1-AS1 increased miR-137 expression, whereas overexpression of miR-137 decreased *TRIM24* expression. Then, expression of miR-137 alleviated the NCK1-AS1 overexpression-induced increased expression of *TRIM24*. In addition, the decreases of cell viability and IC50 induced by NCK1-AS1 knockdown were reversed after adding *TRIM24* in U251/TMZ and A172/TMZ cells.
Conclusion: NCK1-AS1 could increase drug resistance of glioma cells to TMZ by modulating miR-137/*TRIM24* pathway.

Keywords: glioma, miR-137, NCK1-AS1, temozolomide, *TRIM24*

Introduction

Glioma is the most common primary malignant brain tumor in humans, and 40% of patients with glioblastoma have a survival time of less than 1 year.[1] Due to the rapid growth of brain tumors and the specificity of the anatomical position, it is difficult to completely resect the glioma tissues.[2] Three-dimensional (3D) conformal radiotherapy has been found to improve the therapeutic efficiency of glioma, but the radiotherapy effect is not ideal because of the limited radiation dose of normal brain tissue.[3,4] In addition, alkylation agents are also commonly used in the treatment of glioma, but traditional alkylating agents have great toxic side-effects and are prone to drug resistance.[5]

Temozolomide (TMZ) is a new oral dialkylating reagent with a bioavailability close to 100%.[6] It has become a key drug for the treatment of glioma.[6] This drug is quickly absorbed after oral administration and then enters the cerebrospinal fluid through the blood–brain barrier, achieving an effective blood concentration in the central nervous system.[7] Since its inception, TMZ has inhibited the growth of glioma to a certain extent and prolonged the survival of patients.[8,9] However, TMZ-based chemotherapy intrinsic or acquired resistance has increasingly become a limiting factor in the treatment of many patients.[8,9]

Long noncoding RNAs (lncRNAs) are a class of RNA molecules that do not encode functional proteins and are located in the nucleus or cytoplasm. LncRNAs are widely involved in the regulation of various complex diseases in

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humans due to their complex mechanisms, especially in the development of malignant tumors.[10] Some lncRNAs have been shown to play critical roles in the development, progression, metastasis, or invasion of glioma, such as taurine upregulated gene 1 (TUG1), H19, and homeobox A11 antisense RNA (HOXA11-AS).[11–13] In addition, it has been reported that certain lncRNAs, such as metastasis associated lung adenocarcinoma transcript 1 (MALAT1), cancer susceptibility candidate 2 (CASC2), and X-inactive specific transcript (XIST), can affect the sensitivity of glioma cells to TMZ chemotherapy.[14–16] Moreover, Zhang et al. found that decreased expression of noncatalytic kinase 1 antisense RNA 1 (NCK1-AS1) can increase the sensitivity of cervical cancer cells to cisplatin.[17] However, the role of NCK1-AS1 in glioma and its effect on chemotherapeutic drug TMZ are still unclear.

Recent reports have revealed that many lncRNAs regulate the development or occurrence of cancer by regulating microRNAs (miRNAs) and target gene of miRNAs. miR-137 is found to be associated with cancers and regulates glioma cell development.[18] Moreover, lncRNA XIST targets miR-137 to promote glioma cell invasion and viability.[19] In addition, transcription intermediary factor1-α (*TRIM24*), an important member of TRIM family, participates in apoptosis, cell cycle regulation, and other important life processes in cancer cells and is found to be increased in glioma tissue and played a role in chemoresistance.[20] Fang et al. reported that *TRIM24* was a downstream target gene of miR-511 miRNAs, which played a tumor suppressor role in gastric cancer.[21]

Therefore, in this study, the authors established drug-resistance cell model and conducted a series of *in vitro* experiments to investigate the effect of lncRNA NCK1-AS1 on glioma cells' resistance to TMZ and the specific underlying mechanism.

Materials and Methods

Tissue collection

The fresh original glioma tissues, peritumoral brain edema (PTBE), and recurrent tumor tissues were collected from the same patient ($n = 36$, including 23 male and 13 female) during surgery. The patients underwent surgery between December 2017 and January 2019 in The Second Affiliated Hospital of Xi'an Medical University and signed informed consent forms. All experiment protocols were approved by the Ethics Committee of The Second Affiliated Hospital of Xi'an Medical University (Approval No. 2019017).

Cell culture and treatment

Human glioma cell lines U251, A172 and transformed cell line HEK-293 were directly purchased from Procell Life Science & Technology Co., Ltd (Wuhan, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and 1% penicillin–streptomycin (Sigma-Aldrich) at 37°C in a humidified incubator containing 5% CO_2. In addition, resistant U251/TMZ and A172/TMZ cells were screened by continuous treatment of cells with 400 μM TMZ for 4 months.

Cell transfection

For knocking down of NCK1-AS1, small NCK1-AS1 interfering RNA (siRNA) (si-NCK1-AS1) or short-hairpin RNA (shRNA) directed against NCK1-AS1 (sh-NCK1-AS1) was synthesized by GenePharma Co. (Shanghai, China) and transfected into U251, A172, U251/TMZ, or A172/TMZ cells. miR-137 mimics and miR-negative control (NC) were synthesized and transfected into U251 and HEK-293 cells (GenePharma). *TRIM24* overexpression plasmid was synthesized and transfected into U251/TMZ or A172/TMZ cells. Cell transfections were conducted using Lipofectamine 2000 Reagent (Life Technologies Corporation, Carlsbad, CA) following the manufacturer's instruction.

Cell viability assay

After U251/TMZ and A172/TMZ cells were treated with different concentrations of TMZ (0.1, 0.3, 1.2, 2.5, 11.1, 22.2, 100, 200, 900, and 1800 μM, MedChemExpress, New Jersey, NJ) respectively, the precompounded 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazoliumbromide (MTT) solution (10 mg/mL) and Formazan reagent (Beyotime, Shanghai, China) were added to cells and incubated for 4 h at 37°C in a humidified incubator containing 5% CO_2. The spectrophotometer (Thermo Fisher Scientific, Waltham, MA) was used to measure absorbance at 570 nm and half maximal inhibitory concentration (IC50).

Dual luciferase activity assay

Cells were cotransfected with NCK1-AS1-wild type (WT), NCK1-AS1-mature type (MUT), *TRIM24*-WT, or *TRIM24*-MUT and miR-137 mimics or miR-NC by Lipofectamine 2000 reagent. The relative luciferase activities were measured by the Dual-Luciferase® Reporter Assay System Protocol (Promega, Madison, WI) following the manufacturer's information.

RNA pull-down assay

Pierce™ Magnetic RNA-Protein Pull-Down Kit was used to perform RNA pull-down assay (Thermo Fisher Scientific). The cells were lysed with RIPA Lysis Buffer (Beyotime), and magnetic beads were pretreated with 0.1 M sodium hydroxide and physiological saline. The labeled RNA was added to the magnetic beads for 20 min at room temperature, and 10 × protein-RNA binding buffer was added to continue incubation for 60 min (Thermo Fisher Scientific). The RNA-binding protein complex was washed twice by wash buffer and added Elution Buffer to incubate for 20 min. The supernatant was collected for quantitative real-time polymerase chain reaction (qRT-PCR).

Quantitative real-time polymerase chain reaction

The total RNA from cells was extracted in TRIzol Reagent (Life Technologies Corporation). Then, the TaqMan MicroRNA Reverse Transcription Kit (Sigma-Aldrich) and TaqMan Universal Master Mix II (Sigma-Aldrich) were used for qRT-PCR analysis. Fold changes were calculated by relative quantification ($2^{-\Delta\Delta Ct}$) method.[22] The sequences of primers are shown in Table 1.

Table 1. The Quantitative Real-Time Polymerase Chain Reaction Primer Sequences

Primer		*Sequence (5′ to 3′)*
miR-137	Forward	UUAUUGCUUAAGAAUACG CGUAG
	Reward	ACGCGUAUUCUUAAGCAA UAAUU
TRIM24	Forward	CGCCACCCAAGTTGGAGT
	Reward	GCTGGGAACCTCAGTAGT GTCCT
NCK1-AS1	Forward	TTCCCATTTCTCCCAGGTCC
	Reward	R TGGTTACTTTGAGCCTGGC

In situ *hybridization*

The *in situ* hybridization (ISH) Detection Kit III (AP) (Bosterbio, Wuhan, China) was used to perform RNA ISH. Fresh glioma tissue sections were dried for 3 h and fixed with 0.1 M PBS containing 1/1000 diethyl pyrocarbonate (DEPC; Bosterbio) for 20–30 min and digested in 3% citric acid diluted pepsin for 50–120 s at room temperature. The sections were added digoxin labeled oligonucleotide probe diluted by hybridization diluent and mixed overnight at 37–40°C in an incubator. The sections after hybridization were washed twice with saline sodium citrate (SSC; Bosterbio) for 5 min, blocked in blocking solution at 37°C for 20 min, added the alkaline phosphatase-labeled mouse anti-digoxigenin to incubate for 2 h, developed in 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP)/Nitro-Blue-Tetrazolium (NBT) at 37°C for 30–60 min, and finally sealed with a water-soluble seal.

Western blotting analysis

The Cell lysis buffer for Western and IP without inhibitors (Beyotime) was used to extract the protein samples. The proteins were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred onto electro-transferred to polyvinylidene fluoride membranes and blocked with 5% fat-free milk in PBS buffer. The membranes were incubated with primary antibodies against TRIM24 (0.2 mg/mL, ab70560) and actin (0.644 mg/mL, ab179467), which were purchased from Abcam (Cambridge, MA) and diluted 1000 times, and then incubated with horseradish peroxidase-labeled goat anti-mouse (ab6728, 2 mg/mL; Abcam) secondary antibodies. After washing the membranes with 70% Tris (hydroxymethyl) aminomethane (Tris)-HCl (Solarbio, Beijing, China) for 30 min and using glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Abcam) as internal reference, ImageJ software (NIH Image, Bethesda, MD) was used to analyze the level of targeted protein expression.

Statistical analysis

SPSS 19.0 software (SPSS, Inc., Chicago, IL) was used for statistical analysis. The *t*-test was used to compare the two samples, and one-way analysis of variance (ANOVA) was used to compare multiple samples. $p < 0.05$ was considered statistically significant.

Results

NCK1-AS1 expression was increased in glioma tissues

To study the effect of NCK1-AS1 on glioma, the authors first examined the expression of NCK1-AS1 in glioma tissues. The qRT-PCR results in Figure 1A showed that NCK1-AS1 expression in tumor groups was higher than that in PTBE group, especially in the recurrent tumor group ($p < 0.01$). Similarly, ISH results in Figure 1B displayed that NCK1-AS1 expression clearly increased in tumor group compared with PTBE group.

NCK1-AS1 knockdown increased the sensitivity of U251 and A172 cells to TMZ

To investigate the effect of NCK1-AS1 on glioma and TMZ *in vitro*, the authors screened for drug resistance in U251/TMZ and A172/TMZ cells by treatment with 400 μM TMZ. The results in Figure 2A showed that cell viability in drug-resistant U251/TMZ and A172/TMZ cells was higher than that in normal U251 and A172 cells, and cell viability was decreased with increasing TMZ concentration ($p < 0.01$). qRT-PCR results showed that NCK1-AS1 expression was significantly elevated in U251/TMZ and A172/TMZ cells (Fig. 2B, $p < 0.01$). Then, knockdown of NCK1-AS1 was performed by transfection of its siRNA into U251/TMZ and A172/TMZ cells. Figure 2C showed that the significant decreased expression of NCK1-AS1 after the cells was transfected with si-NCK1-AS1. As shown in Figure 2D, MTT assay results indicated that cell viability and IC50 of TMZ were decreased in the si-NCK1-AS1 group compared with si-NC group. Altogether, these results suggested that NCK1-AS1 knockdown recovered the sensitivity of glioma cells to TMZ.

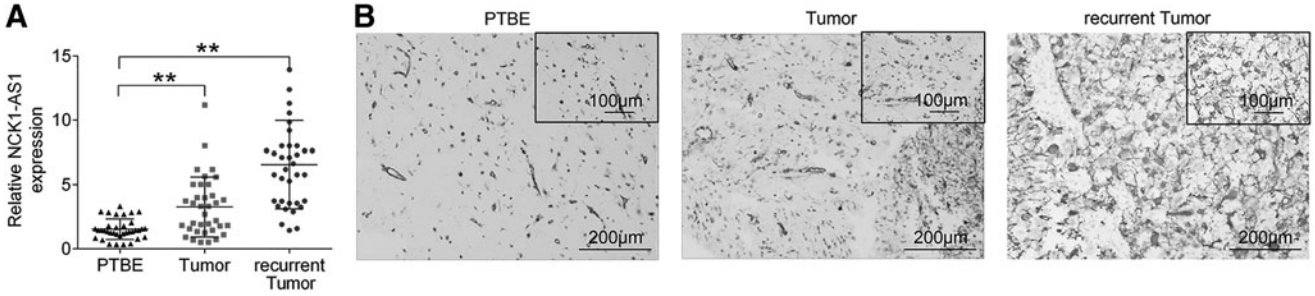

FIG. 1. NCK1-AS1 expression was increased in glioma tissues. The expression of NCK1-AS1 was detected by **(A)** qRT-PCR and **(B)** *in situ* hybridization. $^{**}p < 0.01$. qRT-PCR, quantitative real-time polymerase chain reaction.

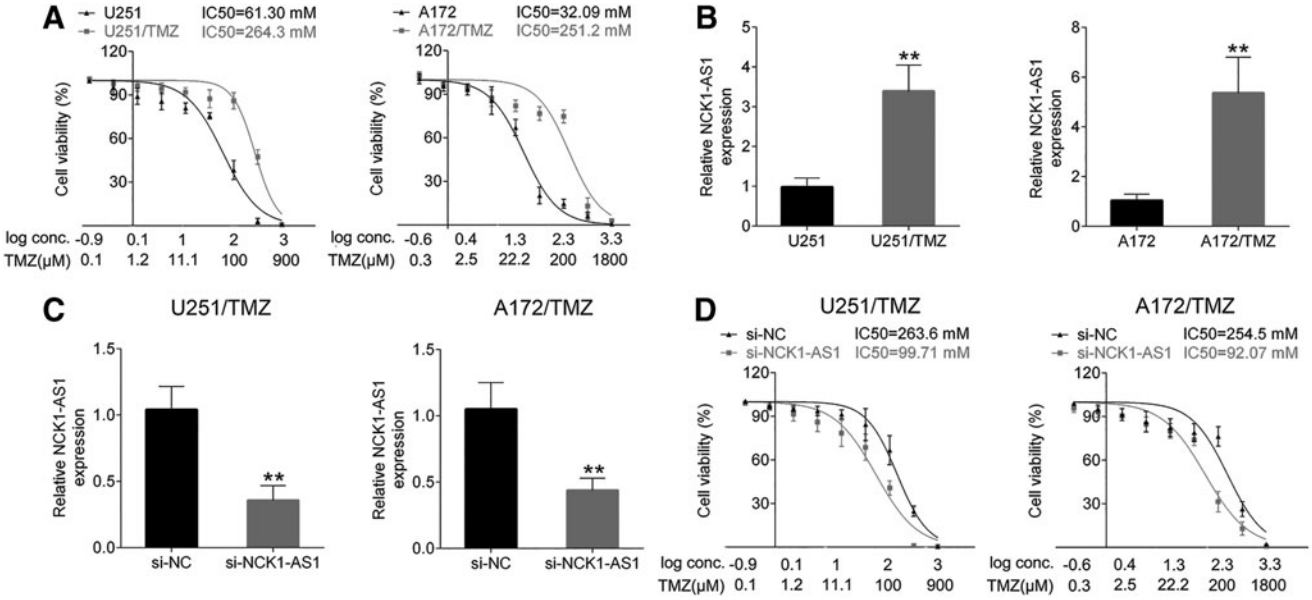

FIG. 2. NCK1-AS1 expression was increased in TMZ-resistant cells, and then knockdown increased the sensitivity of glioma cells to TMZ. **(A)** Cell viability in U251/TMZ and A172/TMZ was detected by MTT assay. **(B)** The expression of NCK1-AS1 was detected by qRT-PCR. **(C)** The expression of NCK1-AS1 was detected by qRT-PCR after adding si-NCK1-AS1. **(D)** The expression of NCK1-AS1 was detected by qRT-PCR after adding si-NCK1-AS1. $^{**}p < 0.01$. TMZ, temozolomide.

NCK1-AS1 negatively regulated miR-137 expression

To further investigate the mechanism of NCK1-AS1 in glioma cells, the authors used the website miRDB (http://mirdb.org/) to predict and screen the possible target genes for NCK1-AS1 and the authors found that there are some binding sites between NCK1-AS1 and miR-137 (Fig. 3A). Then, miR-137 mimics were efficiently transfected into HEK-293 cells (Fig. 3B, $p < 0.01$). Dual luciferase activity assay results in Figure 3C showed that the relative luciferase activity was reduced when miR-137 and NCK1-AS1-WT were cotransfected into the cells compared with cells cotransfected with miR-NC and NCK1-AS1-WT ($p < 0.01$). This result suggested that miR-137 was a target of NCK1-AS1.

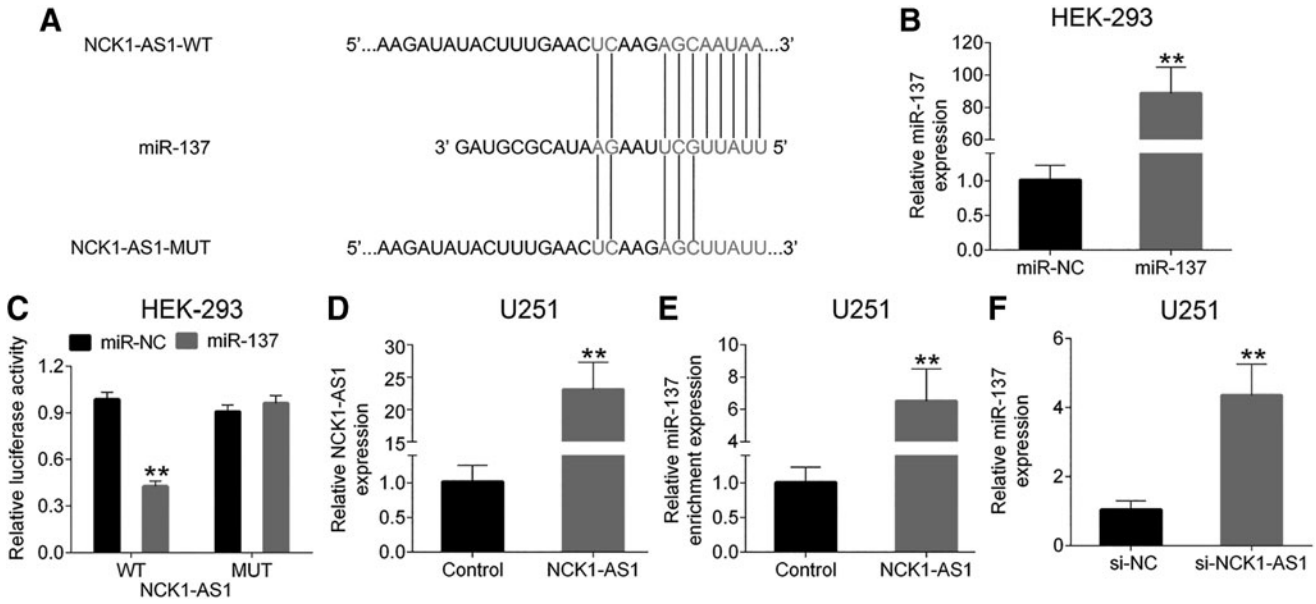

FIG. 3. NCK1-AS1 negatively regulates miR-137 expression. **(A)** The binding sites between NCK1-AS1 and miR-137 were predicted by website. **(B)** The transfection efficiency of miR-137 was detected by qRT-PCR. **(C)** The relationship between NCK1-AS1 and miR-137 was analyzed by dual luciferase activity assay. **(D)** The transfection efficiency of NCK1-AS1 was detected by qRT-PCR. **(E)** The enrichment of miR-137 was detected by RNA pull-down assay. **(F)** The expression of miR-137 was detected by qRT-PCR after adding si-NCK1-AS1. $^{**}p < 0.01$.

To further explore the relationship between NCK1-AS1 and miR-137, U251 cells were transfected with NCK1-AS1 overexpression plasmid (Fig. 3D). The results showed that the expression of miR-137 was increased in the NCK1-AS1 group compared with control group (Fig. 3E, $p<0.01$). In addition, qRT-PCR results in Figure 3F showed that the relative miR-137 expression in the si-NCK1-AS1 group was dramatically higher than that in the si-NC group ($p<0.01$). Hence, these results revealed that miR-137 was a target of NCK1-AS1, and its expression was negatively regulated by NCK1-AS1.

NCK1-AS1 promoted TRIM24 expression by regulating miR-137 expression

Since many miRNAs affect the development of cancer by regulating downstream target genes, the authors predicted the possible target genes of miR-137 by website TargetScan (www.targetscan.org/). Then, the results in Figure 4A showed that there were several targeting sites between miR-137 and its target gene *TRIM24*. Dual luciferase activity assay results confirmed that there was indeed a targeted relationship between miR-137 and *TRIM24*, because luciferase activity was decreased in miR-137 plus *TRIM24*-WT group compared with miR-NC plus *TRIM24*-WT group (Fig. 4B, $p<0.01$), suggesting that *TRIM24* was a target of miR-137. Subsequently, Western blotting results showed that *TRIM24* expression was decreased in miR-137 transfected U251 cells (Fig. 4C, $p<0.01$). On the contrary, *TRIM24* expression was increased when NCK1-AS1 was overexpressed in U251 cells, but this increase was inhibited in NCK1-AS1 plus miR-137 group (Fig. 4D, $p<0.01$). These results demonstrated that miR-137 downregulated and NCK1-AS1 promoted the *TRIM24* expression in glioma cells.

NCK1-AS1 increased the resistance of glioma cells to TMZ by regulating TRIM24

To further study the effects and mechanisms of lncRNA NCK1-AS1 on glioma cells against TMZ resistance, the authors transfected *TRIM24* overexpression plasmid into U251/TMZ and A172/TMZ cells. Figure 5A and B showed that the *TRIM24* expression was clearly increased in the *TRIM24* group ($p<0.01$). Then, MTT assay results showed that the decreases of cell viability and IC50 induced by NCK1-AS1 knockdown were alleviated after overexpression of *TRIM24* in U251/TMZ and A172/TMZ cells (Fig. 5C), suggesting that NCK1-AS1 affected cell viability by regulation of TRIM24 expression.

Discussion

At present, the treatment of glioma is surgery combined with radiotherapy and chemotherapy. However, the treatment effect is not satisfactory, and the recurrence rate and mortality of this disease are very high.[23] Some scholars have found that TMZ can better improve the clinical symptoms of glioma patients and significantly reduces the tumor volume.[9,24] Therefore, TMZ-based chemotherapy is one of the most widely used treatments for glioma.[25,26] In their study, the results showed that cell viability was dramatically decreased in a TMZ dose-dependent manner, suggesting that TMZ has a good anticancer effect. However, studies have found that glioma cells are increasing resistance to TMZ.[8,9] To explore the causes of TMZ resistance, in this study, the authors established cell model in U251/TMZ and A172/TMZ cells to simulate the drug resistance of glioma cells to TMZ *in vitro*. The results confirmed that cell viability was higher in the U251/TMZ and A172/TMZ resistant cells.

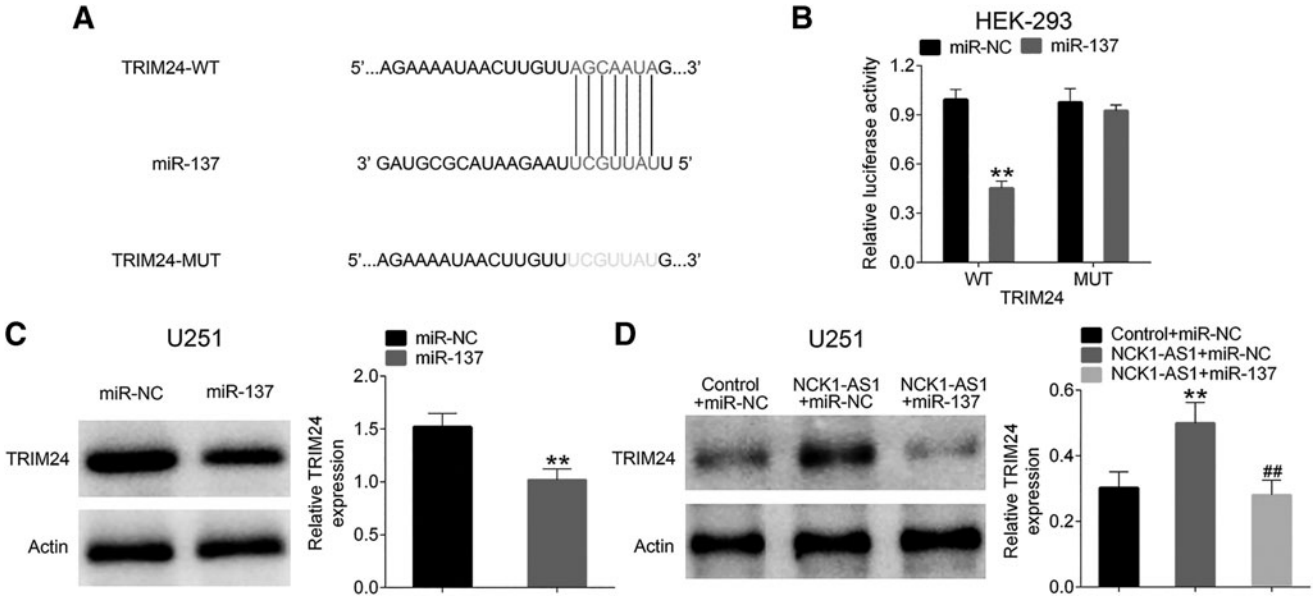

FIG. 4. NCK1-AS1 promoted *TRIM24* expression by regulating miR-137 expression. **(A)** The binding sites between TRIM24 and miR-137 were predicted by website. **(B)** The relationship between TRIM24 and miR-137 was analyzed by dual luciferase activity assay. **(C)** The expression of *TRIM24* was detected by Western blotting. **(D)** The expression of *TRIM24* was detected by Western blotting after adding NCK1-AS1 and miR-137. $^{**}p<0.01$; $^{\#\#}p<0.01$.

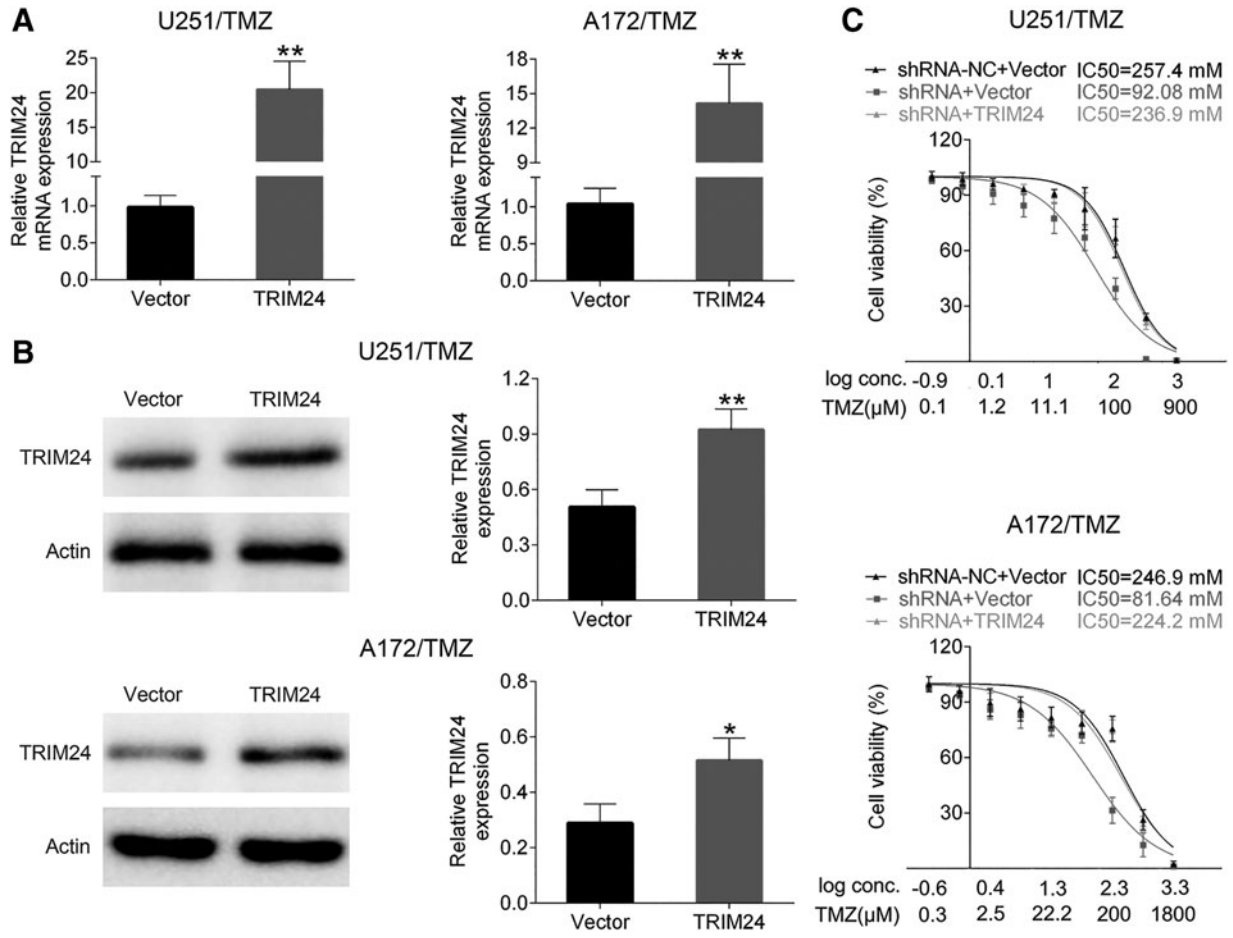

FIG. 5. NCK1-AS1 increased the resistance of glioma cells to TMZ through TRIM24. **(A, B)** The transfection efficiency of TRIM24 was detected by qRT-PCR and Western blotting in U251/TMZ and A172/TMZ cells. **(C)** Cell viability in U251/TMZ and A172/TMZ was detected by MTT assay. $*p < 0.05$; $**p < 0.01$.

In recent years, many studies have shown that aberrantly expressed lncRNAs in tumor tissues can be used as a molecular marker for predicting tumor prognosis.[11,12,14] Li et al. reported that the expression of NCK1-AS1 is increased in cervical cancer tissues and can facilitate cervical cancer cell proliferation.[27] Similarly, this study also found that NCK1-AS1 was highly expressed in glioma tissues, especially in the recurrent tissue group, suggesting that NCK1-AS1 has potential as a prognostic marker for glioma. Moreover, their results showed that NCK1-AS1 expression was upregulated in U251/TMZ and A172/TMZ cells, and the cell viability was reduced when NCK1-AS1 was downregulated, demonstrating that NCK1-AS1 knockdown aggrandized the sensitivity of glioma cells to TMZ. In other words, NCK1-AS1 enhanced the drug resistance of glioma cells to TMZ.

Li et al. also revealed that NCK1-AS1 regulates cell cycle progression induced by miR-6857/cyclin-dependent kinases 1 (CDK1).[27] Then, lncRNA XIST targets miR-137 to promote glioma cell development.[19] Hence, the authors predicted the target genes of NCK1-AS1 through miRDB website and then found and verified the targeting relationship between NCK1-AS1 and miR-137. Bier et al. showed that miR-137 can accelerate cancer stem cell differentiation in glioma by regulating its target gene RTVP-1.[28] In addition, through activating the PI3K/AKT pathway, *TRIM24* increases cancer development and chemoresistance in glioma.[20] Therefore, the authors continued to investigate the relationship between miR-137 and TRIM24, and their results confirmed that *TRIM24* was a target of miR-137. At the same time, the authors also found that NCK1-AS1 increased *TRIM24* expression, while miR-137 inhibited these increases, suggesting that NCK1-AS1 promoted *TRIM24* expression by inhibiting miR-137.

TRIM24 is actually a member of the triple-motif protein family, and its abnormal expression may promote tumor development through a variety of molecular mechanisms.[29,30] A previous study has shown that the expression of TRIM24 from breast epithelial cells to breast invasive ductal carcinoma is gradually increasing, and with the increase of *TRIM24*, the prognosis was worse.[29] *TRIM24* inhibits the occurrence of mouse hepatoma by binding to the same family *TRIM28* and *TRIM33* to form a regulatory complex.[30] However, in their study, the authors found that the decreases in cell viability induced by NCK1-AS1 knockdown were alleviated in TMZ resistant U251/TMZ and A172/TMZ cells, demonstrating that NCK1-AS1 promoted the drug resistance of glioma cells to TMZ by regulating TRIM24 expression.

In conclusion, lncRNA NCK1-AS1 increased the resistance of cells to TMZ by modulating miR-137/*TRIM24* axis in glioma. These results provide new theoretical support for lncRNA NCK1-AS1/miR-137/*TRIM24* regulatory network in the development of glioma. However, due to the singularity of the subjects, it is necessary to further study the role of lncRNAs combined with other chemotherapeutic drugs in glioma, which will provide further evidence for the study of glioma treatment.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Ethics Approval and Consent to Participate

The animal use protocol listed below has been reviewed and approved by the Animal Ethics and Welfare Committee.

Approval No. 2019017

Informed Consent

Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

Authorship Confirmation Statement

M.S.C. and H.K.Z. conceived and designed the experiments, Y.Y.C. and Z.H.Y. analyzed and interpreted the results of the experiments, and F.L.W. and L.Y. performed the experiments.

Disclosure Statement

No competing financial interests exist.

Funding Information

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