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ORIGINAL RESEARCH

Long Noncoding RNA TP73-AS1 Modulates Medulloblastoma Progression In Vitro And In Vivo By Sponging miR-494-3p And Targeting EIF5A2

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Background: Previous studies have shown that P73 antisense RNA 1T (non-protein coding), also known as TP73-AS1, is a long non-coding RNA (lncRNA) and involved in the development of medulloblastoma. However, the regulatory mechanism of lncRNA TP73-AS1 in medulloblastoma was still unclear, the present study was aimed to investigate the detailed functions and the mechanism of TP73-AS1 in regulation of medulloblastoma.

Materials and methods: The levels of TP73-AS1, miR-494-3p, and Eukaryotic initiation factor 5A2 (EIF5A2) were determined using quantitative real-time PCR (qRT-PCR), in situ hybridization (ISH), or Immunohistochemistry (IHC). The function of TP73-AS1 in proliferation, apoptosis, migration, and invasion of medulloblastoma cells was evaluated using cell counting Kit-8 (CCK-8), flow cytometry, and transwell assay, respectively. The protein levels were determined by Western blot. Bioinformatics analysis and dual-luciferase reporter assay, RNA immunoprecipitation (RIP) and pull-down assay were used to search and confirm the target gene of TP73-AS1 and miR-494-3p. The effect of TP73-AS1 knockdown in vivo was detected by animal experiment.

Results: The levels of TP73-AS1 and EIF5A2 were up-regulated, while miR-494-3p expression was down-regulated in medulloblastoma tissues and cells, ELF5A2 was a direct target of miR-494-3p, and miR-494-3p bound to TP73-AS1. The knockdown of TP73-AS1 inhibited cell proliferation, invasion, migration, and promoted apoptosis of medulloblastoma cells, while the miR-494-3p inhibitor abolished the effects of TP73-AS1 knockdown on medulloblastoma cells.

Conclusion: TP73-AS1 positively regulated EIF5A2 expression by sponging miR-494-3p. These findings suggested that TP73-AS1 served as an oncogene and promoted the progression of medulloblastoma.

Keywords: TP73-AS1, miR-494-3p, EIF5A2, medulloblastoma

Introduction

Medulloblastoma (MB) is the most familiar malignant brain tumor in childhood, with high invasiveness and heterogeneity. Medulloblastoma accounts for 15 to 20% of the central nervous system diseases in children.[1,2] About 85% of children with medulloblastoma occur at the age of 5 to 9. Most of the adult patients are 26 to 30 years old, accounting for 43% of adult patients. Most of them are male, and the ratio of male to female is 1.5:1–2:1. Medulloblastoma is composed of many different molecular subtypes. There are significant differences among subtypes in genetics, demography and clinical characteristics. Recently, the mainstream view is

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that medulloblastoma has only four core subtypes: WNT, SHH, Group 3, and Group 4.[3] The identification of different subtypes is not only of great significance for the understanding of medulloblastoma, but also helpful for clinical discrimination and treatment. Recently, the survival rate of patients with medulloblastoma has improved, with a five-year survival rate of 80% (60-70% in high-risk children). However, due to cerebrospinal fluid transmission in some patients, the mortality rate is still very high. Patients with successful treatment often have neurological and endocrine sequelae.[4] Therefore, there is an urgent need for a better alternative treatment to heighten the survival rate and life quality of sufferers.

Along with the development of genomics and genome sequencing technology, more and more lncRNAs have been reported, but their functions need to be further explored. lncRNA is a kind of long transcriptional RNA molecules with size exceeding 200 nucleotides. It is the largest subclass of RNA molecule.[5] LncRNA transcripts do not encode proteins. Recent findings show that 93% of disease-related single nucleotide polymorphism (SNP) is situated in gene control regions or intergenic regions.[6] LncRNA can action through specific linking to DNA or RNA sequences, or uniting with proteins. Kinds of literature have shown that lncRNA plays a vital role in biological processes, like cell growth, apoptosis, migration, and invasion,[7,8] suggesting that lncRNA may be a biomarker and therapeutic target.

LncRNA TP73-AS1 is situated in mankind chromosome 1p36. P73 is one of the members of p53 clan transcription factors. Accumulating studies have delineated the broad biological role of lncRNA on plentiful tumors pathological process, including cell proliferation, tumorigenesis and development.[9] LncRNA TP73-AS1 is carcinogenic in various cancers, for example, brain tumors and esophageal cancer.[10] The knockdown of TP73-AS1 could inhibit the proliferation and induce apoptosis of tumor cells, and exert its function as a tumor inhibitor. Foregoing studies have shown that TP73-AS1 boost the proliferation and restrain apoptosis of medulloblastoma cells.[11] However, the potential mechanism and biologic role of TP73-AS1 in medulloblastoma are still unknown.

MicroRNAs (miRNA) is a non-coding RNA with a stretch of 21–25 nucleotides. It can interact with the target gene at 3′-untranslated regions (3′-UTR) to achieve the purpose of degrading the target gene and blocking the protein expression of the target gene. Therefore, mRNA and its target gene play a major role in the biological process of tumor cells.[12] miR-494-3p is a member of the miR-494 family. Previous researches reported that miR-494-3p could act as an oncomiR. For example, in hepatocellular carcinoma, miR-494-3p could enhance the metastasis of tumor cells,[13] down-regulated expression of miR-494-3p can restrain the proliferation, invasion and promote apoptosis proliferation of glioma cells.[14] While miR-494-3p inhibits the progress and metastasis of prostate cancer through targeting CXCR4.[15] Nevertheless, the functional mechanisms of miR-494-3p in medulloblastoma are still impalpable.

Eukaryotic translation initiation factor (EIF) 5A2 is one of the members of the EIF family. EIF5A2 is an oncogene located on human chromosome 3q25-27.[16] EIF5A2 exists in tumor tissues and is little found in normal tissues,[17] while EIF5A2 protein is up-regulated in ovarian cancer,[18] esophageal cancer,[19] gastric cancer,[20] and colorectal cancer.[21] EIF5A2, as a regulatory molecule, participates in various biological processes of cells, including tumor growth, development, metastasis and epithelial-mesenchymal transition (EMT). It may be a potential prognostic marker.[22,23] Previous researches have shown that EIF5A2 is overexpressed in medulloblastoma, knockdown of EIF5A2 inhibits the proliferation and induces apoptosis of medulloblastoma cells (Dayo), so EIF5A2 plays a key role in the pathogenesis of medulloblastoma.[24]

In the present study, we investigated TP73-AS1 was remarkably increased in medulloblastoma tissues. The knockdown of TP73-AS1 resulted in inhibition of cell proliferation, migration, and invasion. In addition, epithelial-mesenchymal transition (EMT) was also suppressed. Besides, analysis of mechanism revealed that TP73-AS1 modulated EIF5A2 by interacting with miR-494-3p, forming a TP73-AS1/miR-494-3p/EIF5A2 axis.

Materials And Methods

Patient Tissue Specimens

Forty-two specimens of medulloblastoma from 2015 to 2018 were collected at Shangqiu First People's Hospital, the research was supported by the ethics committee of Shangqiu First People's Hospital in accordance with the 1964 Declaration of Helsinki Principles, informed consent were obtained from all participants and signed informed consents were acquired from all patients. All patients had not received any therapies. Medulloblastoma tissues were removed during surgery and rapidly frozen in liquid nitrogen for use.

Cell Culture And Transfection

Human medulloblastoma cell lines (Daoy, D341) were bought from the China Institute of Biochemistry (Shanghai, China). The cells were cultured in Dulbecco's modified eagle medium (DMEM, Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) and then cultured in a humidification incubator containing 5% CO_2 at 37°C.

The TP73-AS1 mimics, small interfering RNA (siRNA) against TP73-AS1 (si-TP73-AS1), TP73-AS1 expression plasmid (pcDNA-TP73-AS1), miR-494-3p mimics, miR-494-3p inhibitor, EIF5A2 mimics, negative control (NC), miR-494-3p inhibitor and scramble negative control (anti-NC) were purchased fromGenePharma (Shanghai, China). Lipofectamine 2000 transfection reagent (Invitrogen) was used to transfer oligodeoxynucleotides and plasmid in medulloblastoma cells with the instruction recommended by the manufacturer, and cells were collected for additional experiments after transfection for 48 h.

In Situ Hybridization (ISH) And Immunohistochemistry (IHC)

ISHwas employed to determine the levels of TP73-AS and miR-494-3p in accordant with the previous describe.[25] IHC was carried out to detect the level of EIF5A2 in line with a protocol elsewhere.[26]

RNA Extraction And RT-PCR

Base on the instruction of the tissue RNA extraction kit (Tiangen Biotechnology Co., Ltd., Beijing, China), total RNA was extracted. Then complementary DNA (cDNA) was synthesized using PrimeScript RT Reagent kit (TaKaRa, Dalian, China) with 1 μg RNA, and the qRT-PCR reaction was carried out by SYBR Green qPCR Master Mix (TaKaRa). The sequence of primers were as follows: TP73-AS1 forward, 5′-GCTCCGTGAACCAACTCG-3′, and reverse, 5′-GCTGCCAAGGGAACTCT-3′; miR-494-3p forward, 5′- GGGTGAAACACACACGGGAA-3′, and reverse, 5′- GGCAGGTCCGAGGT-3′; EIF-5A2 forward, 5′-TTCCAGCACTTACCCTT-3′, and reverse, 5′-TTCCCCTCTATTTTTG-3′; GAPDH forward, 5′-TCGACAGTCAGCCGCATCTTCTTT-3′, and reverse, 5′-ACCAAATCCGTTGACTCCGACCTT-3′; U6 forward, 5′-CTCGCTTCGGCAGCACA-3′, and reverse, 5′-AACGCTTCACGAATTTGCGT-3′. GAPDH and U6 were used as inside parameters to normalize the data, and the mRNA expression level was calculated using $2^{-\Delta\Delta Ct}$.

CCK-8 Assay

Cell proliferation was examined by CCK-8 (Sigma, Saint Louis, MO, USA). After transfection, Daoy and D341 cells (1×10^3/well) were inoculated into 96-well plates, and 10 μL fresh WST-8 reagent (Sigma) with an ultimate density of 0.5 mg/mL was added, following incubation at room temperature for 4 h, and the absorbance of live cells at 450 nm was determined by microplate reader (Thermo Fisher, Waltham, MA, USA).

Transwell Assay

The 24-well transwell chamber (Corning life science, corning, NY, USA) without matrix glue was used to detect the migrationof medulloblastoma cells. Approximately 1×10^5 cells were seeded into the upper chamber, and 500 μL DMEM was added to the lower chamber. After incubation at room temperature for 24 h, the cells were fixed with paraformaldehyde (PFA) for 30 min and stained with crystal violet for 20 min, then observed with an inverted microscope (Thermo Fisher). When the upper chamber was coatedwith matrix glue, and the invasion test could be performed in the same way.

Flow Cytometry Assay

The PI/Annexin V Cell Apoptosis Kit (Sigma) was used to detect cell apoptosis, Daoy and D341 cells were gathered and washed with PBS for 3 times, approximately 1×10^6 cells were resuspended in 200 μL binding buffer, then PI and FITC Annexin V were added to Daoy and D341 cells, and incubated for 15 min at 37°C in the dark, then the cell apoptosis was analyzed by flow cytometry.

Dual-Luciferase Reporter Assay

Online software starBase v2.0 and TargetScan were used to predict the binding sites between miR-494-3p and TP73-AS1 or EIF5A2, respectively. Daoy and D341 cells were inoculated in 24-well plates. The sequences of wild type TP73-AS1 (TP73-WT), mutant TP73-AS1 (TP73-AS1-MUT), wild type EIF5A2 (EIF5A2-WT), and mutant EIF5A2 (EIF5A2-MUT) including the binding sites of miR-494-3p were amplified by PCR and cloned into the pGL3-control luciferase reporter vectors (Promega, Madison, WI, USA), then Daoy and D341 cells were co-transfected with miR-494-3p or miR-control by Lipofectamine 2000 (Invitrogen). After cultured for 48 h, the cells were harvested and the luciferase activity was

checked by dual-luciferase reporter system (Promega) according to the manufacturer's guidance.

RNA Immunoprecipitation Assay

According to the instructions of RIP kit (Millipore, Bedford, MA, USA), Daoy and D341 cells were lysed in RIP lysate. The magnetic beads were preincubated with antibody IgG (Millipore) and anti-Ago2 (Millipore) at 37°C for 1 h. The cell lysates were immunoprecipitated by the immunoprecipitation method and overnight at 4°C, then the detection of purified immunoprecipitated RNA by qRT-PCR.

RNA Pull-Down Assay

According to RNA pull-down assay (Thermo Fisher), biotinylated miRNA-494-3p was transfected into Daoy and D341 cells, with complementary sequences without biotinylated EIF5A2 as the negative control, cells were collected for pull-down assay 48 h later, and enrichment expression was checked by RT-PCR.

Western Blot Assay

The Daoy and D341 cells were harvested and lysed with RIPA buffer (Millipore), then the protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a membrane. The membranewas blocked with 5% skims milk for 1 h at 37°C and incubated overnight at 4°C with anti-EIF5A2 antibody (1:1000, Abcam, Cambridge, MA, USA), E-cadherin (1:1000, Abcam), N-cadherin (1:1000, Abcam), Vimentin(1:1000, Abcam), PCNA (1:1000, Abcam), Caspase3 (1:1000, Abcam) and GAPDH(1:1000, Abcam). Then, the secondary antibody (1:500, Abcam) was added and incubated at room temperature for 1 h. Finally, the membranes were analyzed by the ECL assay (Millipore).

Animal Experiments

Following the agreement of the Animal Care and Experiment Committee of Shangqiu First People's Hospital, and this work was followed by the guidelines of the Institutional Animal Care and Use Committee of Shangqiu First People's Hospital, 12 BALB/C nude mice (4–6 weeks old, 10–15 g, n = 12) were all bought from the Beijing Experimental Animal Center of China (Beijing, China). Mice were subcutaneously injected with 3×10^6D341 cells transfected with lentivirus-mediated TP73-AS1 shRNA (sh-TP73-AS1) or negative control (sh-NC) cells. The tumor volume was measured every 5 days for 5 times. The volume was calculated by length × width × height, for 25 days later, all mice were sacrificed to measure the weight of the tumors.

Statisticanalysis

SPSS software 22.0 (SPSS, Inc, Chicago, IL, USA) was used to analyze all data in this study. The quantitative data were represented as mean±standard deviation. The *t*-tests and one-way analysis of variance were used for comparisons between groups, correlation analysis was conducted by Pearson test. When the *P*-value was less than 0.05, the difference was statistically significant.

Results

The Knockdown Of TP73-AS1 Inhibited Cell Proliferation, Migration, invasion, And Induced Apoptosis Of Medulloblastoma Cells In Vitro

We initially measured the relative transcription level of TP73-AS1 in 42 cases of clinical medulloblastoma patients (Table 1). It was found that the transcription of TP73-AS1 increased in the cancerous tissues of four MB subgroups(WNT, SHH, G3, and G4) when compared with the paired noncancerous tissues (Figure 1A). Moreover, ISH experiment also confirmed that TP73-AS1 level was higher in MB tissues than that in normal tissues (Figure 1A). To validate whether TP73-AS1 affecting the progression of medulloblastoma cells, the si-TP73-AS1 was transfected into Daoy and D341 cells to knockdown TP73-AS1. CCK8 assay showed that the proliferation of Daoy and D341 cells in the si-TP73-AS1 group was significantly lower than that in the control group (Figure 1B and C). Meanwhile, the apoptosis rate of Daoy and D341 cells was

Table 1 Clinical Characteristics Of Chordoma Patients

Clinical Feature	n
Age	
>3	25
≤3	17
Age at diagnosis	
Man	22
Woman	20
MB subgroup	
WNT	5
SHH	12
G3	10
G4	15

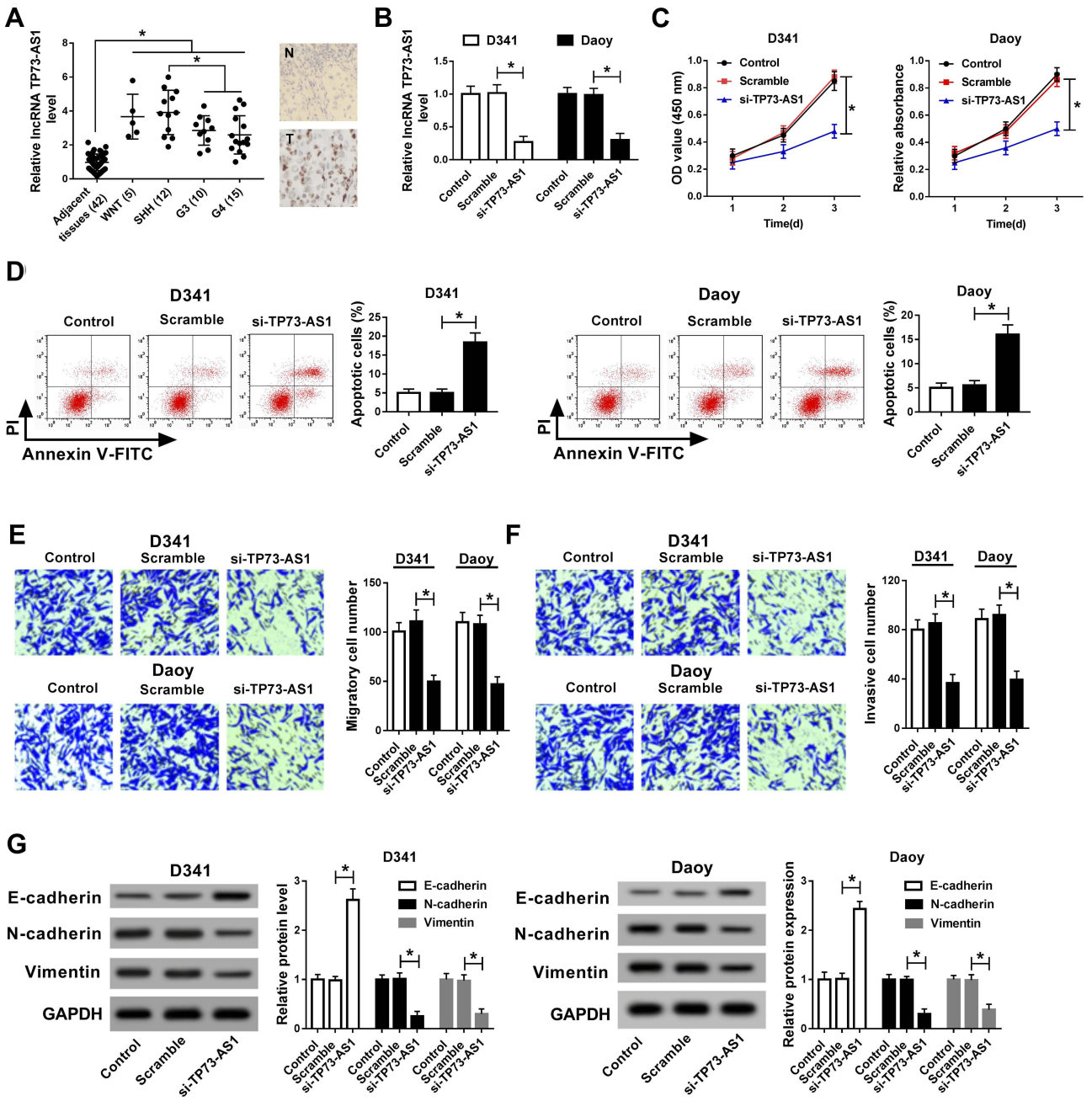

Figure 1 The knockdown of LncRNA TP73-AS1 inhibited cell proliferation, migration, invasion, and induced apoptosis of medulloblastoma cells. (**A**) RT-PCR and ISH revealed the level of TP73-AS1 in 42 medulloblastoma samples. (**B**) RT-PCR showed the expression level of TP73-AS1 after si-TP73-AS1 transfection in Daoy and D341 cells. (**C**) The proliferation ability of Daoy and D341 cells transfected with si-TP73-AS1 was detected by CCK-8. (**D**) The apoptosis ability of Daoy and D341 cells transfected with si-TP73-AS1 was detected by flow cytometry. (**E, F**) transwell assay showed the number of invading cells after si-TP73-AS1 was transfected into Daoy and D341 cells. (**G**) The expression of E-cadherin, N-cadherin and Vimentin proteins in Daoy and D341 cells transfected with si-TP73-AS1 was detected by Western blot.*P< 0.05 compared to control group.

significantly increased by TP73-AS1 knockdown (Figure 1D). Transwell chambers were used to assess the effects of TP73-AS1 on the invasion ability of Daoy and D341 cells. The results showed that fewer Daoy and D341 cells with TP73-AS1 knockdown passed through the membrane compared with control Daoy and D341 cells (Figure 1E and F). Western blot analysis showed that N-cadherin and Vimentin expression were lessened while E-cadherin expression was augmented after TP73-AS1 knockdown (Figure 1G). Usually, Epithelial-to-mesenchymal transition (EMT) is correlative with invasion and metastasis, cell adhesion molecule Ecadherin, a determinant of epithelial cell-cell adhesion, will

be reducedby typical EMT process. However, the expression of Vimentin and Ncadherin, the mesenchymal marker, can be enhanced by the same process. The results verified that TP73-AS1 knockdown inhibits the proliferation and migration in medulloblastoma cells through depressing EMT progress.

TP73-AS1 Targeted miR-494-3p As miRNA "sponge"

Bioinformatics online program (http://starbase.sysu.edu.cn) predicted that TP73-AS1 and miR-494-3p have shared complementary binding sites (Figure 2A). Luciferase reporter showed that TP73-AS1 combined with miR-494-3p at 3′-UTR with molecular binding (Figure 2B). RIP assay presented that TP73-AS1 and miR-494-3p were significantly enriched in AgO2 immunoprecipitation compared with IgG-particles. The results indicated that both TP73-AS1 and miR-494-3p were located in the same RNA-induced silencing complex (RISC) (Figure 2C). Also, the pull-down assay showed that TP73-AS1 directly binds to miR-494-3p in Daoy and D341 cells (Figure 2D). Then the expression of miR-494-3p was detected in Daoy and D341 cells transfected with si-TP73-AS1 and pcDNA-TP73-AS1, respectively. The results showed that the knockdown of TP73-AS1 significantly up-regulated the expression of miR-494-3p, while the overexpression of TP73-AS1 significantly decreased the expression of miR-494-3p in Daoy and D341 cells (Figure 2E). All data showed that TP73-AS1 had binding sites with miR-494-3p, which could specifically inhibit the expression of miR-494-3p and act as a sponge for miR-494-3p.

The Knockdown Of miR-494-3p Restored The Effect Of Si-TP73-AS1 On Proliferation, Apoptosis, Migration, And Invasion In Medulloblastoma Cells

The level of miR-494-3p was investigated by qRT-PCR and ISH, the result revealed that the expression levels of

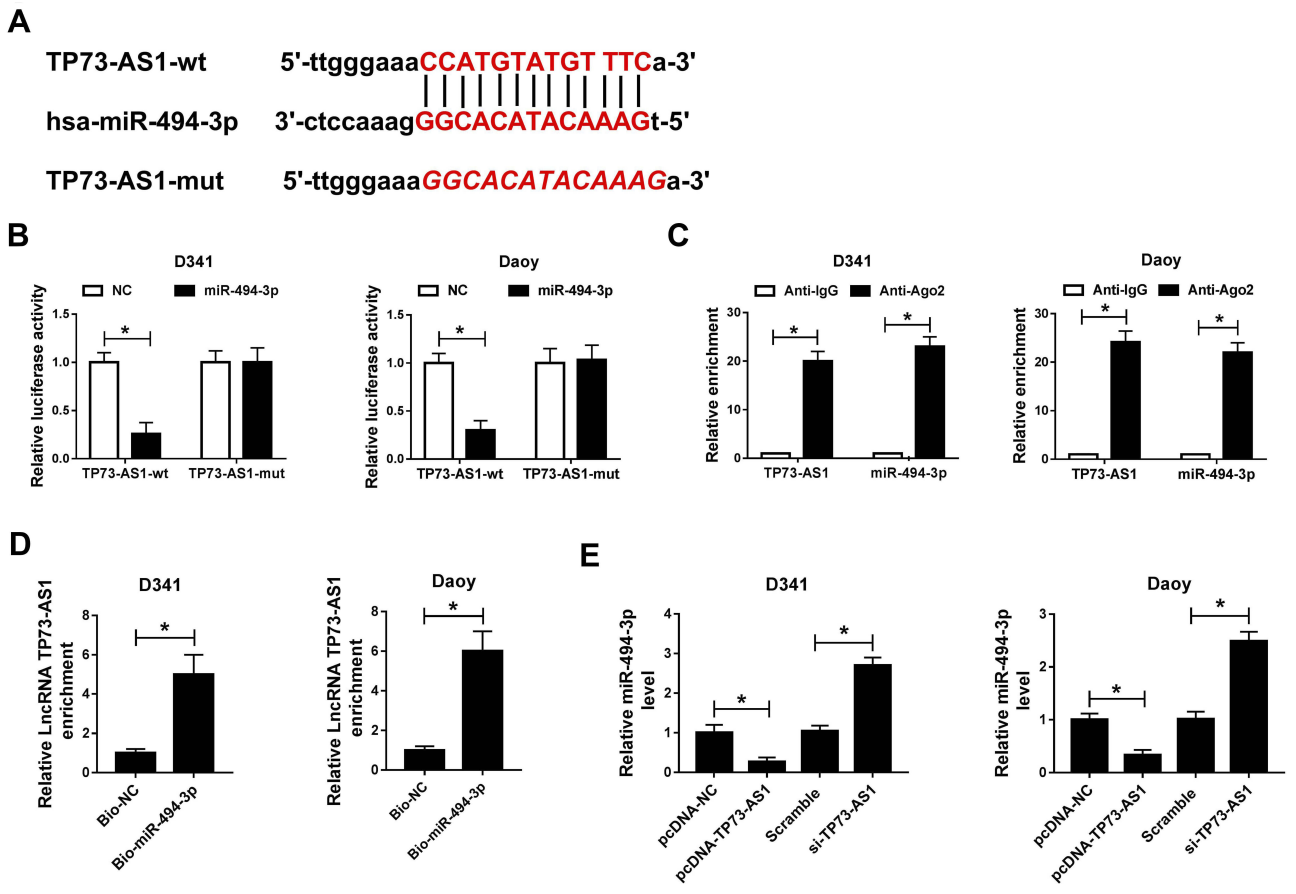

Figure 2 TP73-AS1 targeted miR-494-3p at 3′-UTR with complementary binding sites. (**A**) The predicted binding sequences of TP73-AS1 3′-UTR and miR-494-3p. (**B**) Luciferase reporter assay showed the molecular binding withinTP73-AS1 and miR-494-3p. (**C**) RIP assay wascarried out to explore the exact mechanism of the interaction between TP73-AS1 and miR-494-3p, indicating that both TP73-AS1and miR-494-3p were significantly enriched in Ago2 immunoprecipitate compared to IgG-pellet. (**D**) The relative expression of TP73-AS1which could potentially bind to miR-494-3p was quantified by qRT-PCR after the biotinylated-miR-494-3p pull-down assays in Daoy and D341 cells. (**E**) The expression of miR-494-3p in Daoy and D341 cells transfected with pcDNA-TP73-AS1 and si-TP73-AS1 was detected by RT-PCR. *$P < 0.05$.

miR-494-3p were significantly decreased in the cancerous tissues of four MB subgroups (WNT, SHH, G3, and G4) compared with adjacent normal tissues (Figure 3A). Daoy and D341 cells were transduced into si-TP73-AS1 vector and si-TP73-AS1+anti-miR-494-3p vector to probe the function of miR-494-3p and TP73-AS1. qRT-PCR was operated to determine the expression level. The results showed that si-TP73-AS1 could up-regulate the expression of miR-494-3p and silenced miR-494-3p expression weakened the effect of si-TP73-AS1 (Figure 3B). Besides, cell proliferation indicated that the decreased proliferation of TP73-AS1-downregulated Daoy and D341 cells was enhanced by transfection with miR-494-3p inhibitors (Figure 3C). Next, results of apoptosis and transwell assays were consistent with that of CCK-8 assay (Figure 3D– F). Knockdown of miR-494-3p abolished the effect of si-TP73-AS1 on EMT markers (E-cadherin, N-cadherin, Vimentin) in Daoy and D341 cells (Figure 3G).

miR-494-3p Negatively Regulated The Expression Of EIF5A2

The online software TargetScan also showed that miR-494-3p may bind to 3′-UTR of EIF5A2 (Figure 4A). EIF5A2-WT or EIF5A2-MUT containing wild or mutant miR-494-3p binding sites were constructed, respectively. Dual-luciferase reporter assay showed that co-transfection of mature miR-494-3p and EIF5A2-wt significantly restricted the luciferase activity in Daoy and D341 cells,

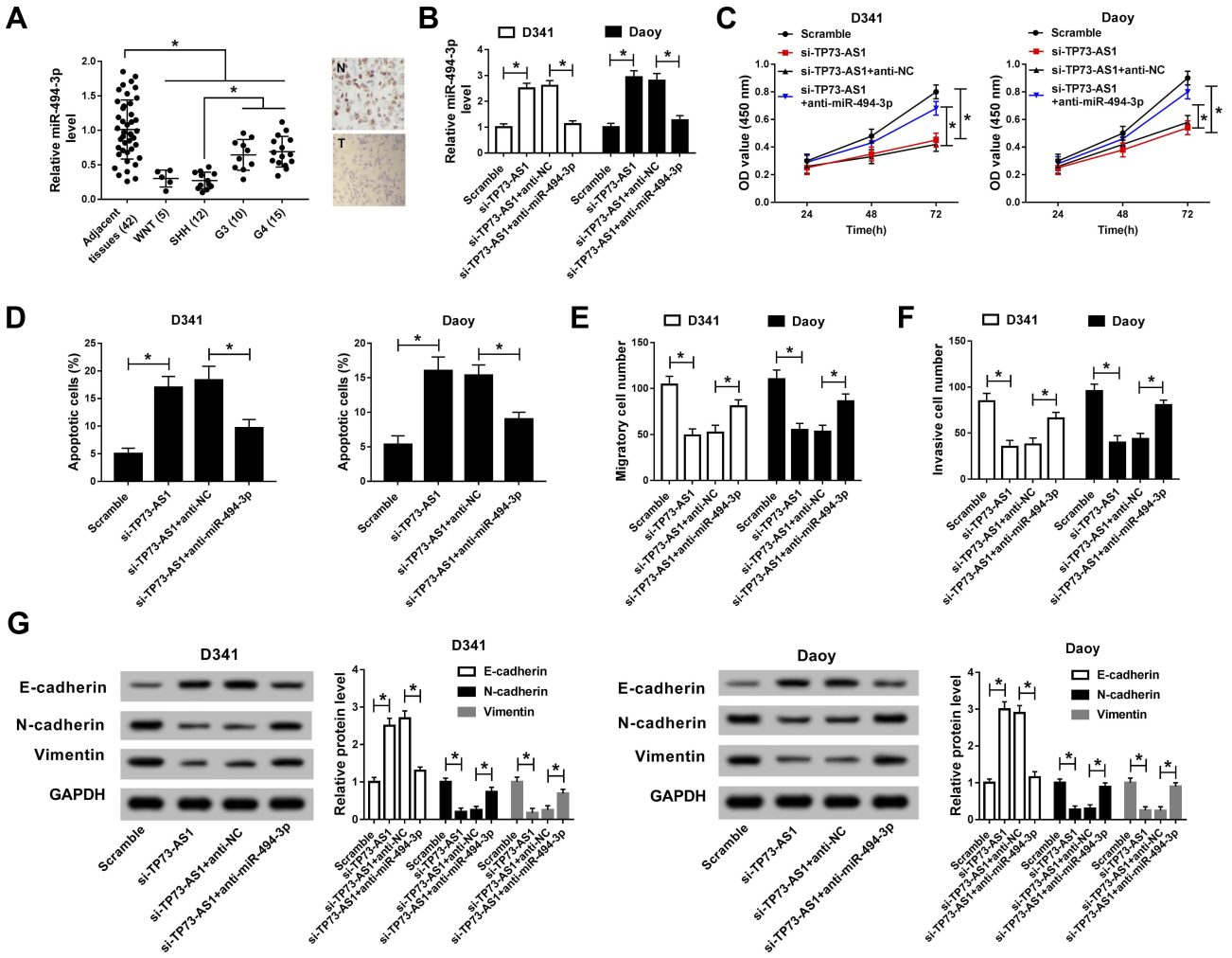

Figure 3 Inhibition of miR-494-3p expression reversed the effects of TP73-AS1 knockdown on proliferation, invasion, and apoptosis of medulloblastoma cells. (**A**) RT-PCR and ISH revealed the expression level of miR-494-3p in 42 medulloblastoma samples. (**B**) RT-PCR showed the expression level of TP73-AS1 after transfected with si-TP73-AS1 or si-TP73-AS1+anti-miR-494-3p in Daoy and D341 cells. (**C**) The proliferation ability of Daoy and D341 cells transfected with si-TP73-AS1 or si-TP73-AS1+ anti-miR -494-3p were detected by CCK-8. (**D**) The apoptosis ability of Daoy and D341 cells transfected with si-TP73-AS1 or si-TP73-AS1+anti-miR-494-3p were detected by flow cytometry. (**E, F**) Transwell assay showed the number of invading cells after si-TP73-AS1 or si-TP73-AS1+anti-miR-494-3p were transfected into Daoy and D341 cells. (**G**) The expression of E-cadherin, N-cadherin and Vimentin proteins in Daoy and D341 cells transfected with si-TP73-AS1 or si-TP73-AS1+anti-miR-494-3p were detected by Western blot. *P< 0.05.

and there was no significant difference in luciferase activity compared with the control group (Figure 4B). RIP assay showed that EIF5A2 was mainly concentrated in the miR-494-3p group (Figure 4C). And Western blot analysis was used to analyze the expression level of EIF5A2 protein in Daoy and D341 cells after transfected with miR-494-3p or anti-miR-494-3p, these results suggested that miR-494-3p significantly inhibited the expression of EIF5A2 in Daoy and D341 cells, and knockdown of miR-494-3p restored EIF5A2 expression (Figure 4D). All the findings verified that miR-494-3p negatively regulates the expression of EIF5A2 in medulloblastoma cells.

miR-494-3p Promoted Proliferation, Migration, Invasion And Inhibited Apoptosis By Affecting The Expression Of EIF5A2

The level of EIF5A2 in medulloblastoma tissues and tumor-adjacent tissues was detected by qRT-PCRand IHC. We found that EIF5A2 expression was significantly increased in the cancerous tissues of four MB subgroups (WNT, SHH, G3, and G4) compared to the adjacent non-tumor tissues (Figure 5A). Western blot analysis suggested that miR-494-3p decreased EIF5A2 protein expression in both Daoy and D341 cells (Figure 5B). miR-494-3p significantly prohibited cell proliferation, promoted apoptosis resistance, next, cell proliferation was induced and apoptosis was inhibited in EIF5A2-overexpressing Daoy and D341 cells (Figure 5C and D). Transwell experiments showed that EIF5A2 overexpression reversed the effect of miR-494-3p on invasion and migration in Daoy and D341 cells (Figure 5E and F). The results suggested that miR-494-3p up-regulation significantly increased the expression levels of N-cadherin and Vimentin, and decreased E-cadherin expression in Daoy and D341 cells, whereas EIF5A2 restoration promoted the activation EMT progression (Figure 5G). All the data showed that EIF5A2 act as an oncogene and inhibitor of miR-494-3p to regulate the biological role of miR-494-3p in medulloblastoma cells.

TP73-AS1 Regulated EIF5A2 By Sponging miR-494-3p In Medulloblastoma Cells

A negative correlation between TP73AS1 and miR-494-3p expression was confirmed by Spearman correlation analysis in medulloblastoma, also we found the negative relevance between EIF5A2 and miR-494-3p, meanwhile TP73AS1 expression was positively correlated with EIF5A2 (Figure 6A–C). Western blot analysis indicated that the knockdown of TP73-AS1 inhibited the expression of EIF5A2, while silencing miR-494-3p inhibited si-TP73-AS1 expression and increased the expression of EIF5A2 (Figure 6D and E). These results suggested that the TP73-AS1 regulated EIF5A2 expression by sponging miR-494-3p.

The Knockdown Of TP73-AS1 Inhibited Themedulloblastoma Tumor Growth In Vivo

To disclose the functional role of TP73-AS1 in medulloblastoma progression in vivo, xenograft models of medulloblastoma were established by injecting Daoy cells into the mice. The knockdown of TP73-AS1 resulted in a notable reduction of tumor volume and weight in vivo (Figure 7A and B). Also, TP73-AS1 knockdown remarkably inhibited the expression levels of TP73-AS1 and EIF5A2 in medulloblastoma xenograft tumors, while enhanced miR-494-3p expression (Figure 7C–E). Proliferating cell nuclear antigen(PCNA) protein as a critical indicator of cell proliferation was diminished by TP73-AS1 knockdown, on the contrary, the caspase-3 protein was markedly high (Figure 7F). These results suggested that knockdown of TP73-AS1 could inhibit medulloblastoma cells proliferation and induce apoptosis in vivo.

Discussion

The refractory nature of medulloblastoma is determined by the high invasiveness of medulloblastoma and the characteristics of metastasis through cerebrospinal fluid. About 40% are diagnosed with distant metastasis. These children need high doses of radiotherapy and chemotherapy through the whole brain, thus causing irreversible harm to the nervous system.[27] With the continuous development of clinical medicine and basic research, the therapeutic effect of medulloblastoma has been well improved. And the 5-year survival rate has reached 30%, and the highest statistics was 80%. However, the prognosis of patients with recurrence and metastasis is much lower than that of the first treatment, even if combined with radiotherapy and chemotherapy.[28] In recent years, the incidence of medulloblastoma in infants and young children is increasing. This high incidence of medulloblastoma urgently requires people to develop novel treatment strategies and chemotherapeutic drugs.

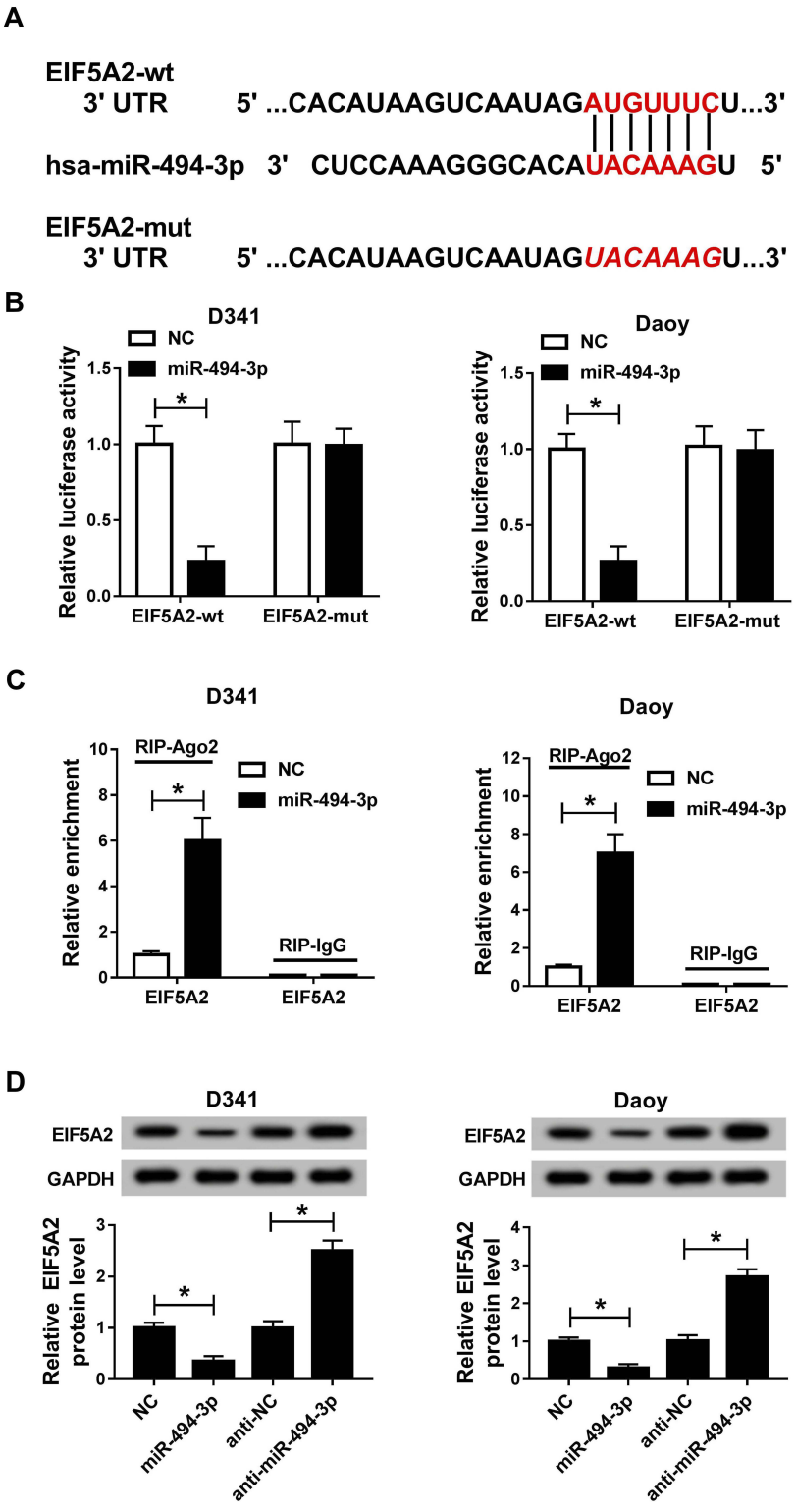

Figure 4 EIF5A2 targeted miR-494-3p with complementary binding sites. (**A**) The predicted binding sites of miR-494-3p in EIF5A2. (**B**) Luciferase reporter assay Daoy and D341 cells simultaneously transfected with miR-494-3p and EIF5A2-WT was significantly reduced, whereas cotransfection of miR-494-3p and EIF5A2-MUT did not change the luciferase activity. (**C**) RIP assay was carried out to explore the exact mechanism of the interaction between EIF5A2 and miR-494-3p, indicating that both EIF5A2 and miR-494-3p were significantly enriched in Ago2 immunoprecipitate compared to IgG-pellet. (**D**) The expression of EIF5A2 decreased in Daoy and D341 cells transfected with miR-494-3p, and the expression of EIF5A2 increased significantly in Daoy and D341 cells transfected with anti-miR-494-3p. *P< 0.05.

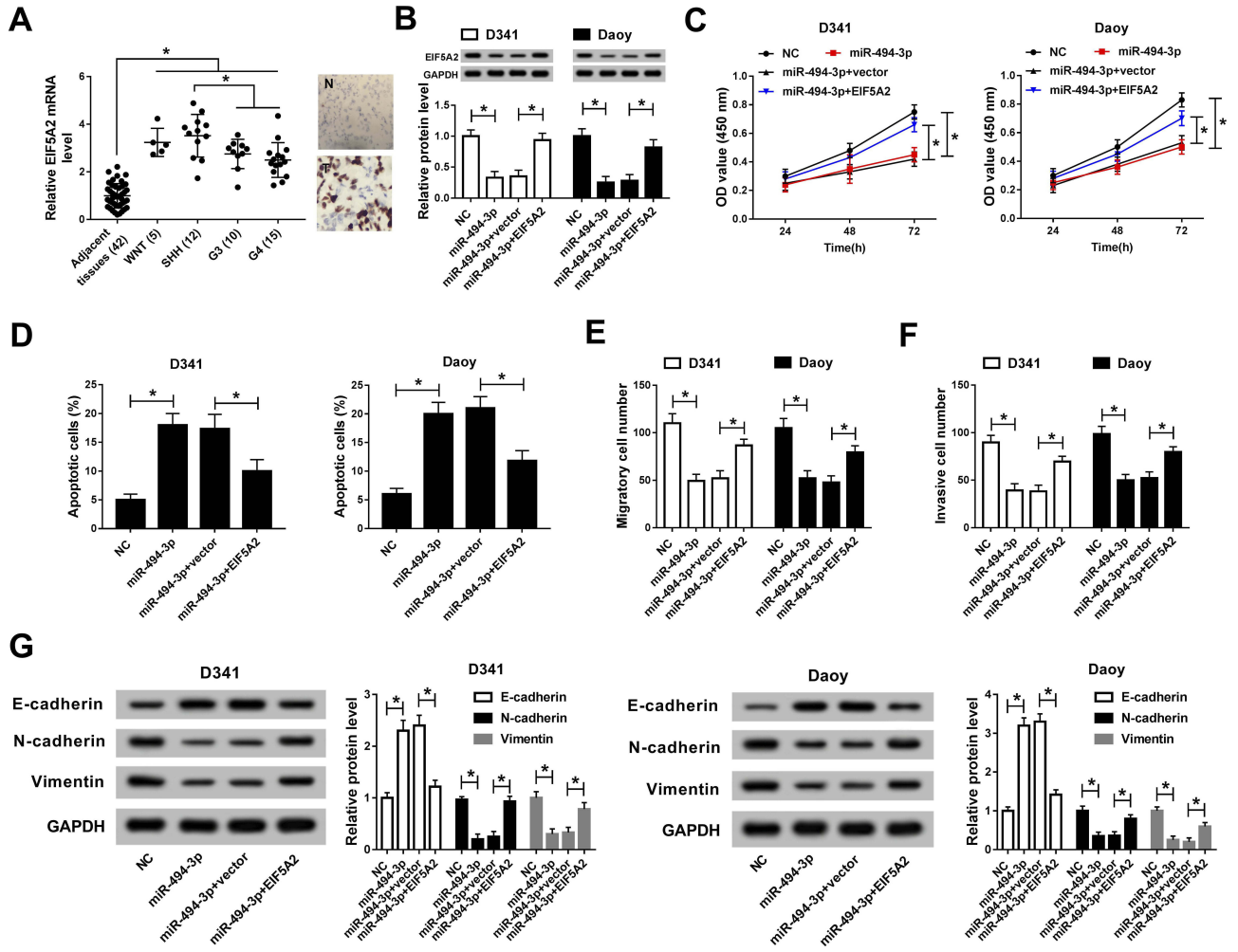

Figure 5 EIF5A2 overexpression reversed the effects of miR-494-3p knockdown on proliferation, invasion, and apoptosis of medulloblastoma cells. (**A**) RT-PCR and IHC revealed the expression level of EIF5A2 in 42 medulloblastoma samples. (**B**) Western blot showed the expression level of EIF5A2 in Daoy and D341 cells after transfected with miR-494-3p or miR-494-3p+EIF5A2. (**C**) The CCK-8 assay was used to detect cell viability of Daoy and D341 cell transfected with miR-494-3p or miR-494-3p+EIF5A2 at the indicated time points. (**D**) Flow cytometry was used to evaluate apoptosis of Daoy and D341 cell transfected with miR-494-3p or miR-494-3p+EIF5A2. (**E, F**) Transwell assay showed the invaded cell number in Daoy and D341 cells transfected with miR-494-3p or miR-494-3p + EIF5A2. (**G**) The expression of E-cadherin, N-cadherin and Vimentin proteins in Daoy and D341 cells transfected with miR-494-3p or miR-494-3p + EIF5A2 were detected by Western blot.*P< 0.05.

Long non-coding RNA is an important regulatory factor of many diseases and occupies a crucial position in the occurrence and evolution of human cancer.[9] It has been reported that TP73-AS1 is highly expressed in hepatocellular carcinoma, and restrains the expression of miR-200a through competing between HMGB1 and miR-200a.[29] Our results showed that TP73-AS1 in clinical medulloblastoma tissues was up-regulated, which was consistent with the previous conclusion.[11] The knockdown of TP73-AS1 suppressed the proliferation, invasion and EMT formationin medulloblastoma cells, suggesting that TP73-AS1 is a tumorigenesis promoter. Besides, EIF5A2 was confirmed to be a target gene of TP73-AS1, and was up-regulated in medulloblastoma tissues, there was an obviously positive correlation between TP73-AS1 and EIF5A2.

MiRNA is highly conservative and stable, and inhibits protein translation by binding to mRNAs 3′-UTR, which becomes a biomarker and therapeutic target for many diseases.[30] miRNA-494-3p has been identified as a novel type of cancer-associated microRNAs, which is considered as an oncogene and involved in the evolution of tumors.[14] In our current research, we observed that miRNA-494-3p could contribute to cell growth and suppress apoptosis in medulloblastoma cells, which is consistent with the previous study.[31] To further research the biological function of miRNA-494-3p, we confirmed that TP73-AS1 and EIF5A2 were the targeted genes of the miRNA-494-3p by RIP and pulldown assay, down-regulated miR-494-3p expression in medulloblastoma cells could recover the expression of TP73-AS1 and EIF5A2. It is well known that EIF5A2 promotes cell

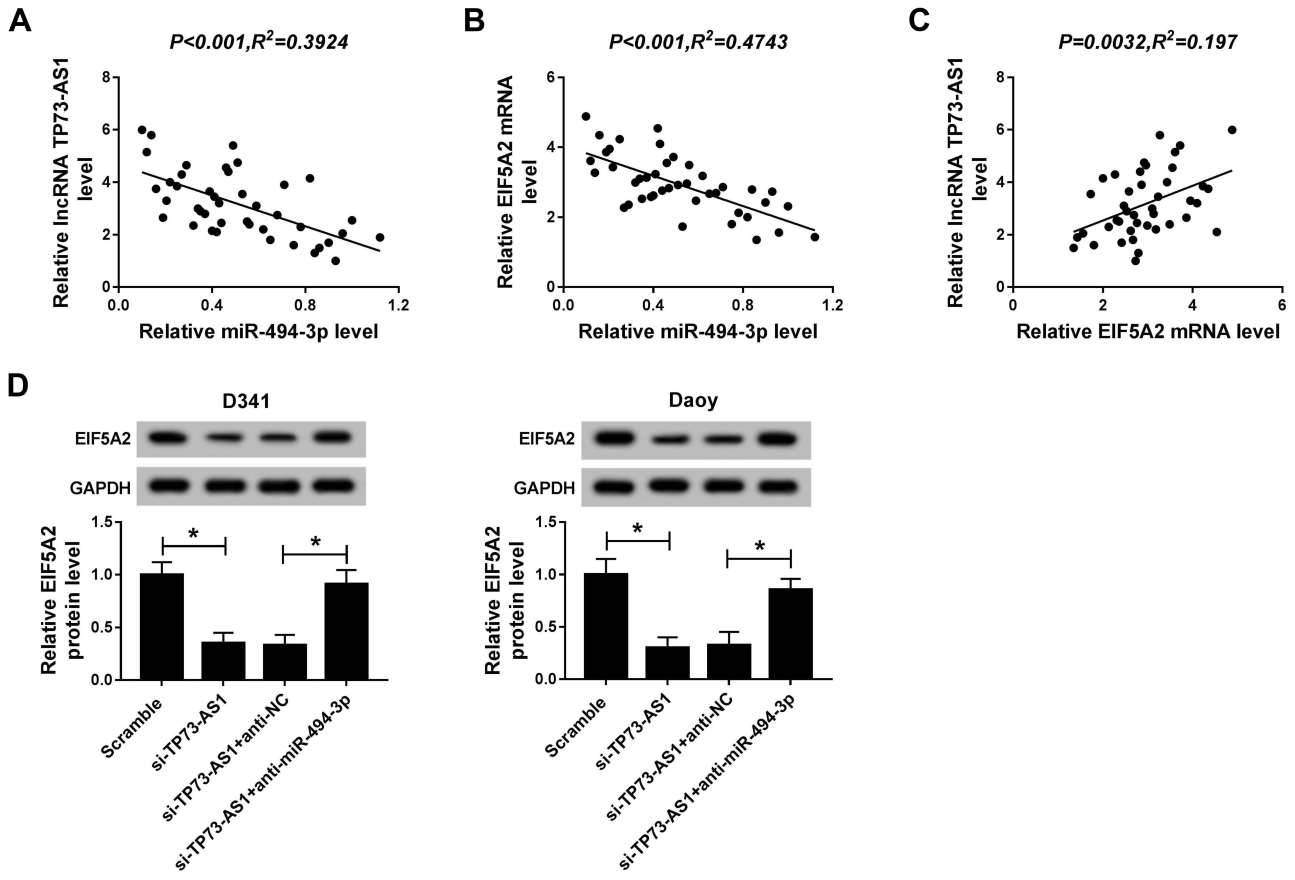

Figure 6 TP73-AS1 targeted EIF5A2 by sponging miR-494-3p in medulloblastoma cells. (**A, B, C**) Pearson's correlation showed TP73-AS1 was negatively correlated with miR-494-3p, miR-494-3p was negatively correlated with EIF5A2 mRNA, and EIF5A2 mRNA was positively correlated with TP73-AS1. (**D**) The protein level of EIF5A2 in si-TP73-AS1 and si-TP73-AS1+anti-miR-494-3p Daoy and D341 was detected by Western blot. *P< 0.05.

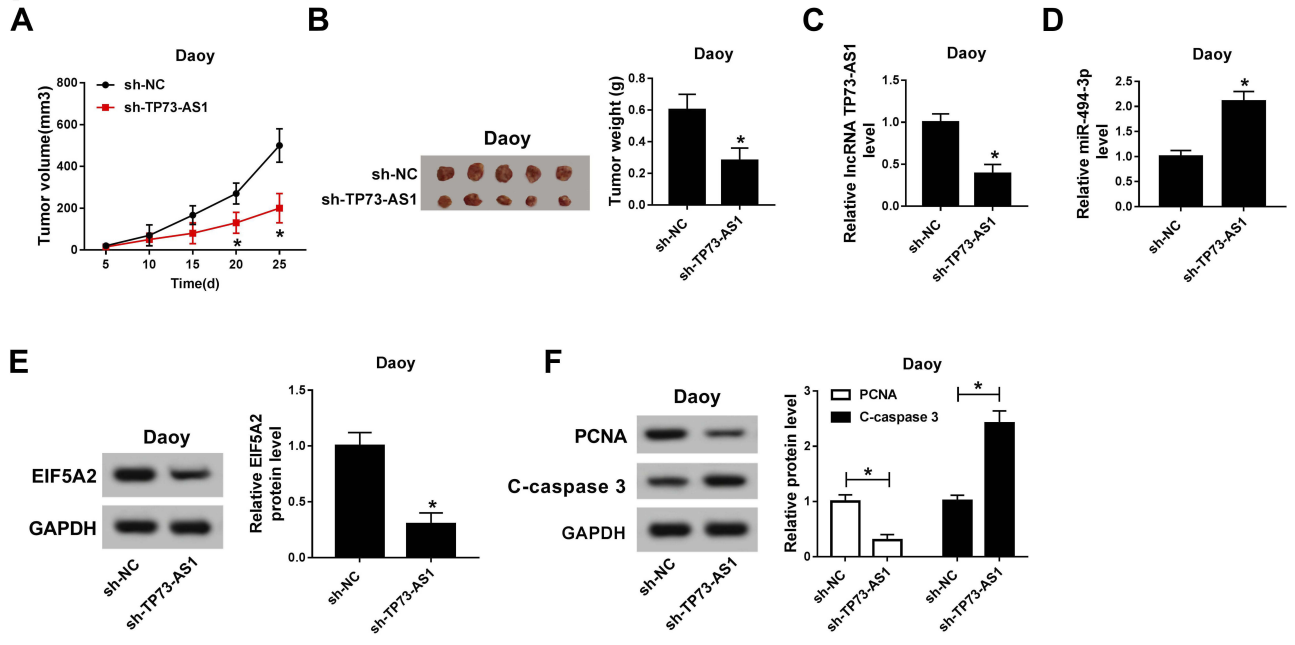

Figure 7 TP73-AS1 knockdown suppressed the proliferation and invasion ability in vitro and inhibited tumor growth in vivo. (**A, B**) The tumor volume and weight in mice injected with Daoy cells transfected with lentivirus-mediated transfection shRNA or controls. (**C, D**) Detection of the expression of TP73-AS1 and miR-494-3p in Daoy cells transfected with sh-TP73-AS1 by RT-PCR. (**E, F**) Western blot analysis revealed the protein levels of EIF5A2, PCNA and C-caspase3 in Daoy cells transfected with sh-TP73-ASI. *P< 0.05.

proliferation and migration and acts as a biological predictor of poor prognosis.[32–34] Also, TP73-AS1 and EIF5A2 were negatively correlated with miR-494-3p, in the research, it was demonstrated that miR-494-3p could target EIF5A2, dual-luciferase reporter assay and RIP assay confirmed the connection of miR-494-3p and EIF5A2. The expression of EIF5A2 was robustly decreased by miR-494-3p. Later, the rescue assays were performed to delineate the impacts of the changes of TP73-AS1, miR-494-3p and EIF5A2 on the proliferation, migration, invitation, apoptosis and EMT process of medulloblastoma cells. In the end, we found that TP73-AS1 was activated by EIF5A2 and modulated the progression of medulloblastoma via sponging miR-494-3p to enhance the expression of EIF5A2.

Furthermore, other lncRNAs also regulate cell progression in medulloblastoma. For example, lncRNA CCAT1 expedites the proliferation and invasion of medulloblastoma cells through the MAPK pathway,[35] and CRNDE promotes the growth of medulloblastoma cells in vivo and in vitro by preventing cell cycle progression and inhibiting cell apoptosis.[36] Although TP73-AS1 and EIF5A2 relations with medulloblastoma have been reported, the regulation mechanism about them in medulloblastoma is unclear. We aim to expound the knockdown TP73-AS1 in medulloblastoma organization and expression of cell lines. Consequently, our research is the first to reveal the influence of TP73-AS1/miR-494-3p/EIF5A2 on the biological progression of medulloblastoma.

Conclusion

In summary, the lncRNA TP73-AS1 was highly expressed in medulloblastoma tissues and cell lines, TP73-AS1 acts as a potential oncogene and regulates proliferation, migration, invasion, and apoptosis by targeting miR-494-3p/EIF5A2 in medulloblastoma cells. Further understanding of TP73-AS1 /miR-494-3p/EIF5A2 axis associated mechanisms would provide a possible future therapy for medulloblastoma patients.

Disclosure

The authors declare that there are no conflicts of interest in this work.

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