AMOTL2-knockdown promotes the proliferation, migration and invasion of glioma by regulating β-catenin nuclear localization

XINGJIE CHEN[1-3*], YALIN LU[1-3*], GAOCHAO GUO[4*], YU ZHANG[1-3], YAN SUN[5], LIANMEI GUO[1-3], RUOHONG LI[1-3], YANG NAN[1-3], XUEJUN YANG[1-3], JUN DONG[6], XUN JIN[7-10] and QIANG HUANG[1-3]

[1]Department of Neurosurgery, Tianjin Medical University General Hospital; [2]Key Laboratory of Post-Trauma Neuro-Repair and Regeneration in Central Nervous System, Ministry of Education and Tianjin City; [3]Tianjin Key Laboratory of Injuries, Variations and Regeneration of Nervous System, Tianjin 300052; [4]Department of Neurosurgery, Henan Provincial People's Hospital, Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450003; [5]Department of Neurosurgery, The Affiliated Yantai Yuhuangding Hospital of Qingdao University, Yantai, Shandong 264000; [6]Department of Neurosurgery, The Second Affiliated Hospital of Soochow University, Suzhou, Jiangsu 215004; [7]Tianjin Medical University Cancer Institute and Hospital; [8]National Clinical Research Center for Cancer; [9]Key Laboratory of Cancer Prevention and Therapy; [10]Tianjin's Clinical Research Center for Cancer, Tianjin 300060, P.R. China

Received January 9, 2021; Accepted April 8, 2021

DOI: 10.3892/or.2021.8090

Abstract. Glioblastoma multiforme (GBM) is the most prevalent type of malignant cancer in the adult central nervous system; however, its mechanism remains unclear. Angiomotin-like 2 (AMOTL2) is a member of the motin family of angiostatin-binding proteins. It has been reported as an oncogene in cervical and breast cancer, but its association with glioma remains unknown. The aim of the present study was to investigate AMOTL2-regulated processes in glioma cell lines using extensive *in vitro* assays and certain bioinformatics tools. These results revealed that AMOTL2 was downregulated in high-grade glioma cells and tissues, with patients with glioma exhibiting a high AMOTL2 expression having a higher survival rate. The results of the glioma cell phenotype experiment showed that AMOTL2 suppressed GBM proliferation, migration and invasion. In addition, immunoblotting, co-immunoprecipitation and immunofluorescence assays demonstrated that AMOTL2 could directly bind to β-catenin protein, the key molecule of the Wnt signaling pathway, and regulate its downstream genes by regulating β-catenin nuclear translocation. In conclusion, the present study demonstrated that AMOTL2 inhibited glioma proliferation, migration and invasion by regulating β-catenin nuclear localization. Thus, AMOTL2 may serve as a therapeutic target to further improve the prognosis and prolong survival time of patients with glioma.

Correspondence to: Professor Qiang Huang, Department of Neurosurgery, Tianjin Medical University General Hospital, 154 Anshan Road, Heping, Tianjin 300052, P.R. China
E-mail: huangqiang209@163.com

*Contributed equally

Key words: angiomotin-like 2, glioblastoma, bioinformatics, Wnt/β-catenin signaling, nucleus translocation

Introduction

Glioblastoma multiforme (GBM) is the most prevalent malignant tumor in the adult central nervous system, with a median survival time of only 12-14 months following initial diagnosis, and a 5-year survival rate of only 5.5% (1,2). It is characterized by an extremely poor prognosis, high recurrence rate and short median survival time following initial diagnosis. According to pathological histology, primary brain tumors have been classified by the World Health Organization (WHO) into four grades (grades I-IV), with GBM (grade IV) as the most malignant glioma (3). To date, the main treatments of glioma are surgical resection, radiotherapy and chemotherapy; however, obtaining an optimal effect is challenging (4). Therefore, an improved understanding of the underlying mechanisms of the occurrence and progression of glioma are essential for the development of diagnostic markers and novel effective treatments.

Angiomotin (AMOT) belongs to the motin family of angiostatin-binding proteins (5). The motin family, also known as AMOTs, consists of three members: AMOT, AMOT-like 1 (AMOTL1) and AMOTL2 (5). Among these, AMOTL1 and AMOTL2 belong to human protein sequences and have similar structure. Members of the motin family are a type of adaptor proteins mainly distributed in the cytomembrane, cytoplasm or nucleus, and have a higher expression in the endothelial cells of capillaries and in larger vessels of the placenta (6). During the formation of new blood vessels, angiostatin can inhibit the tube formation and migration of endothelial cells

toward growth factors, while members of the motin family can regulate this effect to maintain normal physiological functions (7). Although there is a high similarity between the members of AMOTs, their various functions remain mostly unknown (8). Functionally, AMOTs have been revealed to directly regulate the initiation and progression of cancer through various pathways. For example, AMOTs can interact with core protein Yes-associated protein (YAP)1 in the Hippo signaling pathway, which leads to its inhibition, and bind to AKT pleckstrin homology domain to block membrane localization of AKT and negatively regulate the AKT signaling pathway (9,10). At present, AMOTL1 has been primarily reported as an oncogene in cervical and breast cancer, while it could suppress tumorigenesis in GBM (6).

The Wnt signal transduction cascade is a major regulator of development throughout the animal kingdom and a key signaling pathway mediating development and stemness (11). In adult mammals, the Wnt/β-catenin signaling pathway is also a key driver of most types of tissue stem cells. Recently, with the progress of sequencing technology and the deepening of the understanding of structural characterization of cancer genomes, certain Wnt signaling pathway components have been revealed to play key roles in various growth-related pathologies and cancers (12). Wnt/β-catenin signaling regulates tumorigenesis and progression mainly through the transcriptional regulation of its downstream genes, including cyclin D1 (13), Myc (14), MMP7 (15), Snail (16) and Sox9 (17), thus resulting in cancer cell proliferation, migration, invasion and cancer stem cell property maintenance (18,19). Therefore, delineation of the mechanisms of Wnt/β-catenin signaling in cancer may provide novel insights into the development of targeted cancer therapies.

Currently, there are few studies on the mechanism of action of AMOTL2 in glioma, however, our study found abnormal expression of AMOTL2 in glioma and its ability to combine with β-catenin. In order to further understand the mechanism, in the present study, a series of experiments including western blotting, qPCR, and immunofluorescence (IF) staining were conducted to explore the effect of AMOTL2 on glioma and the relationship between AMOTL2 and β-catenin.

Materials and methods

Public data collection. Gene expression and survival analysis data were obtained from the Chinese Glioma Genome Atlas (http://www.cgga.org.cn; dataset ID: mRNAseq_693) (20,21) and The Cancer Genome Atlas (TCGA; http://cancergenome.nih.gov/) databases. Gene Ontology (GO) (22) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (23) analyses were conducted by DAVID (https://david.ncifcrf.gov/gene2gene.jsp) (version 6.8) (24) using data downloaded from the CGGA database. The GO and KEGG analysis of genes were drawn using the ggplot2 package of R software (https://cran.r-project.org/web/packages/ggplot2/index.html).

Patients and specimens. The GBM specimens were collected from the Tianjin Medical University General Hospital (Tianjin, China). The inclusion criteria was a pathologically confirmed diagnosis of GBM. The age range of the patients was from 30 to 60 years and the sex ratio was 1:1. Informed consent for the use of these tissues and data in the present study was obtained from the patients, their immediate family members or their guardians. The study was approved by the Ethics Committee of Tianjin Medical University General Hospital (Tianjin, China).

Immunohistochemistry (IHC). Glioma tissues of clinical patients were fixed with 4% formaldehyde for 24 h at room temperature, paraffin-embedded at 54˚C for 4 h and cut into 4-μm paraffin sections. Following heating at 60˚C for 2 h, the paraffin sections were deparaffinized by xylene and rehydrated in graded ethanol. Following antigen extraction with 0.1% sodium citrate buffer (pH 6.0) at 95˚C, the tissue sections were quenched of endogenous peroxidase activity with 3% $H_2O_2 \cdot dH_2O$ and blocked with 5% goat serum at room temperature for 30 min. Next, the tissue sections were incubated with antibodies against AMOTL2 (1:200; cat. no. 36101; Signalway Antibody LLC) overnight at 4˚C and then incubated with HRP-labeled goat anti-rabbit antibody (product no. ZB-2010; ZSGB-BIO, Inc.) at room temperature for 15 min. Following three washes by PBS for 5 min/wash, the slides were stained by diaminobenzidine (cat. no. DA1010; Beijing Solarbio Science & Technology Co., Ltd.) for 3 min at room temperature and counterstained by hematoxylin (cat. no. B3671; APeXBIO Technology LLC) for 10 min at room temperature. Finally, graded ethanol was used to dehydrate these tissues, which were then sealed using neutral balsam and visualized using a fluorescence microscope (cat. no. IX81; Olympus Corporation; magnification, x20).

Cell lines. Human GBM cell lines U251 and glioblastoma of unknown origin U87MG were purchased from American Type Culture Collection. Cells were cultured at 37˚C in a humidified 5% CO_2 atmosphere with Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Inc.) containing 10% FBS (Thermo Fisher Scientific, Inc.). These cell lines underwent mycoplasma tests and were authenticated by Short Tandem Repeat (Beijing Microread Genetics Co., Ltd).

Lentiviral vectors with AMOTL2 short hairpin (sh)RNA and overexpressed plasmids of AMOTL2. The lentiviral vectors with AMOTL2 and control shRNA were purchased from Shanghai GeneChem Co., Ltd. A 2nd generation system was used and the interim cell line used was 293T (Shanghai GeneChem Co., Ltd.). The packaging vector: Envelope was 3:1 and the duration of transfection was 48-72 h. The 4-μg lentivirus was transfected into the target cells (the MOI was ~1-3) when the cell density was 70-80%. After maintaining for 12-16 h, the medium with lentivirus was replaced to fresh DMEM containing 10% FBS. After infection, cells were selected using a 2 μg/ml puromycin solution (Thermo Fisher Scientific, Inc.) and maintained using a 1 μg/ml puromycin solution to create stable infection cell lines to conduct follow-up experiments. Subsequent experiments were performed 72 h after transfection.

AMOTL2 plasmid was created using Shanghai GeneChem Co., Ltd., and the overexpressed plasmids of AMOTL2 were purchased from Addgene, Inc. The 723 ng/μl plasmid was transfected into target cells using Lipofectamine 3000 (Thermo Fisher Scientific, Inc.) at room temperature for 72 h and subsequent experiments were conducted.

Western blotting. Cell samples were lysed in RIPA lysis and extraction buffer (cat. no. 89901; Thermo Fisher Scientific, Inc.) containing protease inhibitor (cat. no. 36978; Thermo Fisher Scientific, Inc.). After evaluating the concentration using a BCA protein assay kit, protein samples were denatured by heating at 100˚C and mixed with loading buffer. The protein samples were separated on 10% SDS-PAGE gels at 10 μg per lane and electrophoretically transferred to a PVDF membrane (EMD Millipore). Following blocking with 5% skimmed milk for 1 h at 37˚C, incubation with primary antibodies overnight at 4˚C and incubation with secondary antibodies for 1 h at room temperature, the membrane was exposed through G:BOX (Syngene Europe) using immobilon western chemiluminescent HRP substrate (cat. no. WBKLS0500; EMD Millipore) to achieve the target proteins. The primary antibodies used in this study were as follows: Anti-AMOTL2 rabbit monoclonal antibody (1:1,000; product no. 43130; Cell Signaling Technology, Inc.), anti-β-catenin rabbit monoclonal antibody (1:5,000; product code ab32572; Abcam), anti-cyclin D1 rabbit monoclonal antibody (1:1,000; product no. 2922; Cell Signaling Technology, Inc.), anti-c-Myc rabbit monoclonal antibody (1:1,000; product no. 18583; Cell Signaling Technology, Inc.), anti-GAPDH mouse monoclonal antibody (1:2,000; cat. no. 40493; Signalway Antibody LLC), anti-histone H3 Rabbit monoclonal antibody (1:1,000; product no. 4499; Cell Signaling Technology, Inc.). The secondary antibodies used in this study were as follows: Goat anti-rabbit IgG H&L (HRP) (1:5,000; product code ab6721) and goat anti-mouse IgG H&L (HRP) (1:5,000; product code ab6789; both from Abcam).

Reverse transcription-quantitative (RT-q)PCR assay. Total RNA from cells was extracted by TRIzol (cat. no. 15596018; Thermo Fisher Scientific, Inc.) and reverse-transcribed using GoTaq® Reverse Transcription system (cat. no. A3500; Promega Corporation), according to the manufacturer's instructions. Moreover, 2X SYBR Green qPCR Master mix (low ROX) was purchased from Bimake, and the reaction conditions used were according to the manufacturer's instructions (denaturation: 95˚C for 15 sec; annealing/extension: 60˚C for 30-60 sec; 40 cycles). Comparative quantification was performed using the $2^{-\Delta\Delta Cq}$ method with GAPDH as the endogenous control (25). All primers were synthesized by Beijing Tianyi Huiyuan Bioscience & Technology Inc. The primer sequences used were as follows: GAPDH forward, 5'-GGAGCGAGATCCCTCCAAAAT-3' and reverse, 5'-GGCTGTTGTCATACTTCTCATGG-3'; AMOTL2 forward, 5'-TGGAGAAGACCATGCGGAAC-3' and reverse, 5'-CTTCTCTTGCTCCTGCTGCT-3'.

Colony formation assay. Cells were generated by seeding 500 cells/well in 6-well plates and subsequently incubating for 10-14 days at 37˚C. Cells were fixed with 4% paraformaldehyde for 10 min at room temperature and stained with 0.5% crystal violet blue for 10 min at room temperature. The cell colonies were observed and images were captured by camera and colonies with >10 cells were counted using a fluorescence microscope (cat. no. IX81; Olympus Corporation; magnification, x10).

Cell Counting Kit-8 (CCK-8) cell viability assay. A CCK-8 assay was conducted using a CCK-8 kit (cat. no. PA5-84814; Thermo Fisher Scientific, Inc.) at 1-5/6 days. GBM cells were seeded at $3x10^3$ cells/well in 96-well plates and cultured for 24 h at 37˚C. The CCK-8 mixture was configured and added to each well, and the cells were incubated for 1 h at 37˚C. Finally, the optical density value was measured using a microplate reader (BioTek Instruments, Inc.) at 450 nm.

Wound-healing assay. Glioma cells were seeded at $4x10^5$ cells/well in 6-well plates and incubated for 12 h. A total of 4 vertical straight scratches were drawn in each well using a 10-μl sterile pipette tip. Plates were washed using PBS and incubated with serum-free medium. Images of the scratches were captured at 0, 12 and 24 h by a fluorescence microscope (cat. no. IX81; Olympus Corporation; magnification, x4) to evaluate the rate of wound healing. Gap width analysis was performed with ImageJ 1.43 software (National Institutes of Health) and the maximum confluence percentage reached 93%.

Transwell migration and Matrigel® invasion assays. A total of $2x10^4$ cells were seeded in the upper chambers of 24-well plates (pore size: 3 μm; Corning, Inc.), incubated with 200 μl DMEM without FBS in the upper chambers, and 500 μl DMEM with 10% FBS was added at the bottom of the 24-well plates. Following incubation for 24 h at 37˚C, cells were fixed with 4% paraformaldehyde for 10 min and stained with 0.5% crystal violet blue for 5 min at room temperature. Images of the Transwell were captured by a fluorescence microscope (cat. no. IX81; Olympus Corporation; magnification, x10). For the Matrigel® invasion assay, $5x10^4$ cells were added to the bottom membrane of the upper chamber, which was coated with 100 μl Matrigel® (1:5 dilution with DMEM without FBS) for 1 h at 37˚C, and the aforementioned steps as in the migration assay were conducted.

Co-immunoprecipitation (Co-IP) assay. Cells were lysed in 400 μl IP lysis buffer (cat. no. 87787; Thermo Fisher Scientific, Inc.), supplemented with complete protease inhibitor cocktail (CAS no. 329-98-6; Beijing Solarbio Science & Technology Co., Ltd.). The lysates were incubated with 40 μl Protein A Agarose (cat. no. AS046; ABclonal Biotech Co., Ltd.) and appropriate antibodies were placed in a rotating incubator overnight at 4˚C. Then, the cells were centrifuged at 5,939 x g at 4˚C for 5 min, to remove the supernatant and the precipitation was washed three times with PBS. After boiling with 5X SDS-PAGE loading buffer (cat. no. Top2225; Biotopped Life Sciences, Inc.) for 10 min at 100˚C, western blotting was conducted to further analyze the immunoprecipitated samples. The antibodies used in Co-IP assay in this study were as follows: Anti-AMOTL2 rabbit monoclonal antibody (1:200; product no. 43130; Cell Signaling Technology, Inc.), anti-β-catenin rabbit monoclonal antibody (1:200; product code ab32572; Abcam).

Immunofluorescence (IF) staining. Glioma cells were seeded on cover slides in a 24-well plate chamber with 500 μl medium containing 10% FBS incubated for 24 h at 37˚C. Cells were then fixed with 4% paraformaldehyde for 30 min, permeabilized by PBS with 0.1% Triton X-100 (PBST) for 5 min, and blocked with 1% BSA in PBST at room temperature. Immunostaining was conducted using the primary antibodies against β-catenin (1:50; cat. no. ab32572; Abcam) overnight

at 4˚C and goat anti-rabbit secondary antibodies conjugated with Alexa Fluor® 594 (1:200; cat. no. A11037; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. Images were captured using a confocal microscope (x20).

β-catenin pathway inhibitor treatment. β-Catenin pathway inhibitor (XAV-939; cat. no. S1180) was purchased from Selleck Chemicals. The AMOTL2-knockdown U87MG and U251 cells were treated with 20 μM of XAV939 for 24 h.

Statistical analysis. All quantitative data are expressed as the mean ± SD. Statistical analysis was performed with GraphPad Prism 8 software (GraphPad Software, Inc.). Unpaired Student's t-test was used to determine the statistical significance of the data between two experimental groups, while one-way ANOVA was used to analyze the significance of multiple group comparisons. Tukey's post hoc test was performed after ANOVA. All statistical analyses were two-sided, and different cut-off values, ($P<0.05$, $P<0.01$, $P<0.001$), were considered to indicate statistically significant differences. All experiments were repeated at least in triplicate.

Results

AMOTL2 expression is decreased in GBM, as compared with low-grade glioma. To assess the differences in AMOTL2 expression between different glioma grades, data on the expression of AMOTL2 in glioma from TCGA and CGGA databases were analyzed. Data from TCGA database revealed that AMOTL2 expression was significantly decreased in WHO IV grade glioma, as compared with WHO II and III grade glioma (Fig. 1A). Similarly, data from the CGGA database indicated that AMOTL2 expression was significantly decreased in GBM, as compared with low-grade glioma (LGG; Fig. 1A). Notably, the overall survival analysis of these data indicated that glioma patients with a high AMOTL2 expression had a high survival rate (Fig. 1B). Furthermore, data from the CGGA database on isocitrate dehydrogenase (IDH) indicated that the AMOTL2 expression level of the IDH mutant (MUT) was significantly higher than that of the IDH wild-type (WT) (Fig. 1A). Consistently, the results of survival analysis revealed that patients with IDH MUT had a longer survival rate than patients with IDH WT (Fig. 1B). Next, IHC of clinical specimens from patients with glioma was performed to further examine the expression level of AMOTL2. The results indicated that the expression of AMOTL2 was decreased as the malignancy grades increased (Fig. 1C). In conclusion, it was revealed that AMOTL2 was clearly decreased in GBM, and may act as an inhibitor of glioma to prolong the survival time of patients with glioma.

AMOTL2 inhibits the proliferation, migration and invasion of glioma cells. To identify the effect of the differential expression of AMOTL2 in glioma progression, the classic glioma cell lines U87MG and U251 were selected for subsequent tests. First, lentiviral vectors with AMOTL2 shRNA and overexpressed plasmids of AMOTL2 were used to transfect the U87MG and U251 cells to induce AMOTL2-knockdown or -overexpression. Western blotting and RT-qPCR were used to examine the efficiency of the infection of shRNAs and plasmids in U87MG and U251 cell lines (Figs. 2A and B, and S1A and B). Colony formation assays revealed that the downregulation of AMOTL2 significantly promoted colony formation, and AMOTL2-overexpression inhibited colony formation both in U87MG and U251 cells (Figs. 2C and S1C). Consistently, as revealed in Fig. 2D, the result of CCK-8 assays also demonstrated that AMOTL2 overexpression could inhibit the proliferation of U87MG and U251 cells.

It is well-known that the migrating and invasive abilities of tumor cells cause tumor metastasis and poor prognosis. In the present study, following the incubation of cells for 24 h in 6-well plates, 4 vertical straight scratches were drawn. Next, the wound-healing rate was detected at 0, 12 and 24 h to examine the migrating ability of cells, which was revealed to be significantly inhibited by AMOTL2 overexpression and increased following AMOTL2 silencing (Figs. 2E and S1F). Next, a Transwell assay with or without Matrigel® was conducted to further detect the migrating and invasive abilities of glioma cells. The results revealed that the knockdown of AMOTL2 clearly promoted the migration and invasion of U87MG and U251 cells. On the contrary, the overexpression of AMOTL2 significantly reduced the cell migratory and invasive abilities of the two cell lines (Figs. 2F, and S1D and E). In conclusion, AMOTL2 was revealed to inhibit proliferation, migration and invasion in glioma cells.

KEGG and GO enrichment analyses of AMOTL2. To further explore the mechanism of AMOTL2, functional and pathway enrichment analyses were performed using data from the CGGA database by 'ggplot2' R language package. The GO analysis results revealed that changes in AMOTL2 biological processes (BP) were significantly enriched in 'DNA-templated transcription' and 'DNA-templated regulation of transcription' (Fig. 3A). Changes in molecular function (MF) were mainly enriched in 'protein and DNA binding' (Fig. 3B). The results of cellular component (CC) analysis revealed that AMOTL2 was primarily enriched in the 'nucleus', 'cytoplasm' and 'nucleoplasm' (Fig. 3C). Furthermore, the results of KEGG pathway analysis revealed that the changes of AMOTL2 were mainly enriched in the 'Wnt', 'cell cycle' and 'FoxO signaling pathways' (Fig. 3D).

Downregulation of AMOTL2 promotes β-catenin nuclear localization and activates the Wnt/β-catenin signaling pathway. Based on the results of GO and KEGG analysis, whether AMOTL2 regulates the Wnt/β-catenin signaling pathway via protein binding was next examined. Co-IP assay between AMOTL2 and β-catenin was performed, and the results revealed that AMOTL2 and β-catenin proteins could mutually combine (Fig. 4A). It was consistent with our hypothesis that AMOTL2 probably plays its role in tumor suppression by directly binding to β-catenin. Subsequently, western blotting demonstrated that AMOTL2-knockdown could increase the expression of certain downstream genes of the Wnt/β-catenin signaling pathway, such as c-Myc and cyclin D1, to activate Wnt signaling; however, no change was observed in the expression of β-catenin (Fig. 4B). This indicated that the function of AMOTL2 in the regulation of Wnt pathway genes activity occurs other than via direct action on the β-catenin destruction complex. It has been reported that, in addition to

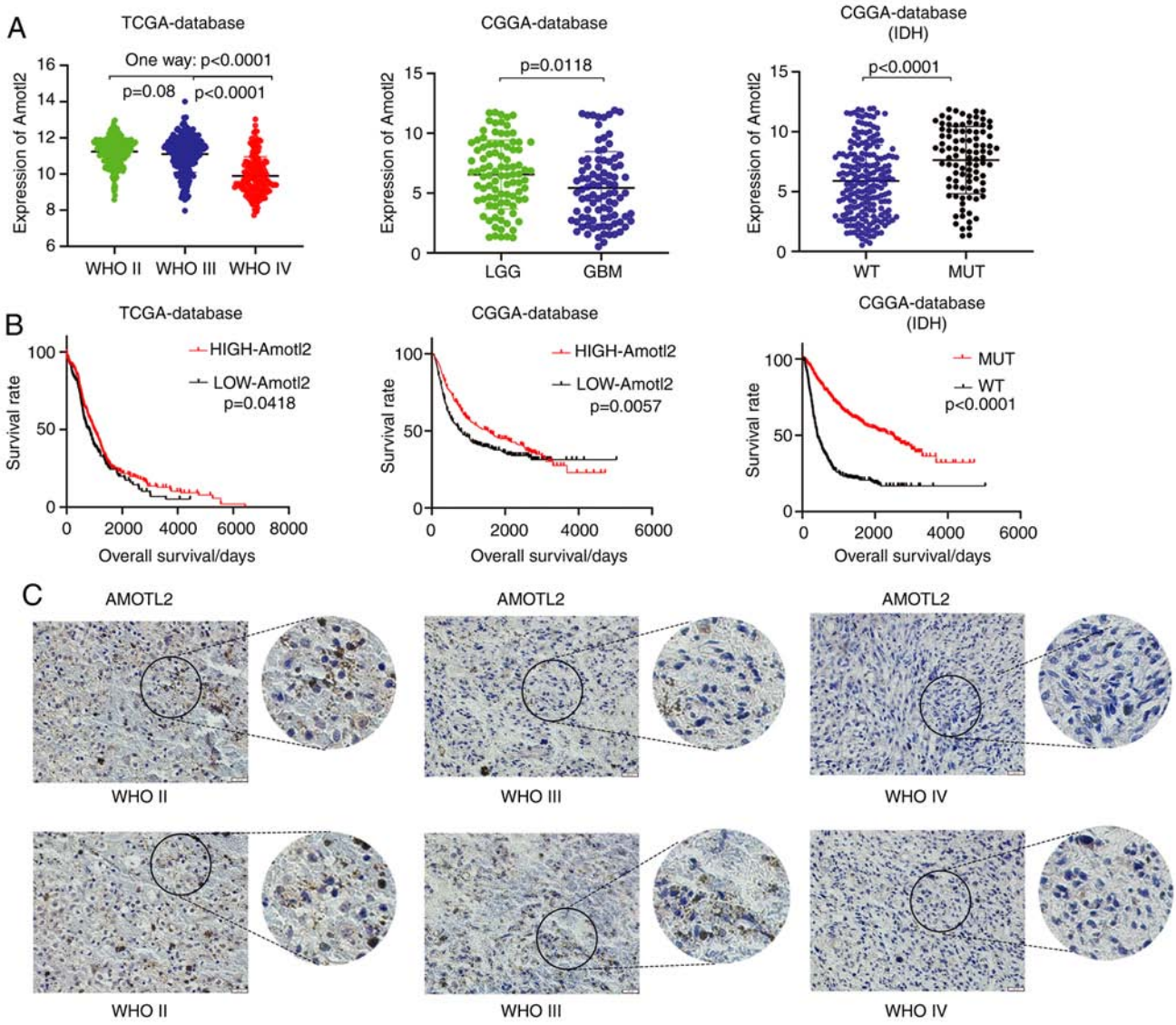

Figure 1. Expression level of AMOTL2 is reduced in GBM, as compared with low-grade glioma. (A) Gene expression analysis was performed using data from TCGA (left) and CGGA (middle and right) databases. (B) Overall survival analysis was performed using data from TCGA (left) and CGGA (middle and right) databases. (C) Immunohistochemical staining of protein AMOTL2 was performed in grade WHO II, III and IV clinical specimens from glioma patients. AMOTL2, angiomotin-like 2; GBM, glioblastoma; TCGA, The Cancer Genome Atlas; CGGA, Chinese Glioma Genome Atlas.

the β-catenin destruction complex, the activation of the Wnt pathway was also regulated by the distribution of β-catenin in the nucleus and cytoplasm (26). Therefore, to further identify the mechanism of AMOTL2 in the Wnt signaling pathway, the nuclear and cytoplasmic localization of β-catenin in control and AMOTL2-silenced cells were next examined.

First, western blotting of nuclear and cytoplasmic proteins in AMOTL2-silenced and control cells was performed. AMOTL2 was revealed to significantly increase the nuclear distribution and decrease the cytoplasmic distribution of β-catenin proteins (Fig. 4B). Next, immunofluorescence assays were performed to further illustrate the effect of AMOTL2 on β-catenin protein localization. AMOTL2 downregulation was revealed to clearly promote the nuclear transfer of β-catenin protein, both in U87MG and U251 cells (Fig. 4C).

In conclusion, these results indicated that AMOTL2 could prevent β-catenin from translocating to the nucleus by directly binding to the β-catenin protein.

Wnt/β-catenin pathway inhibitor can reverse the AMOTL2-knockdown-induced proliferation, migration and invasion of glioma. It is unclear whether the regulatory function of AMOTL2 over proliferation, migration and invasion of glioma is realized through the Wnt/β-catenin signaling pathway. Therefore, the AMOTL2-knockdown U87MG and U251 cells were treated with Wnt/β-catenin pathway inhibitor XAV939 with 20 μM for 24 h. The efficiency of the Wnt/β-catenin pathway inhibition was identified by western blotting, and the results showed that the treatment group exhibited a clearly lower β-catenin protein expression, as compared with the control (Figs. 5A and S2A). Colony formation assays revealed that the AMOTL2-silencing-induced increased colony formation ability decreased following XAV939 treatment (Figs. 5B and S2B). Notably, it was revealed by CCK-8 assay that XAV939 reduced the proliferation of AMOTL2-knockdown cells (Figs. 5C and S2C). These results indicated that the Wnt pathway inhibitor XAV939

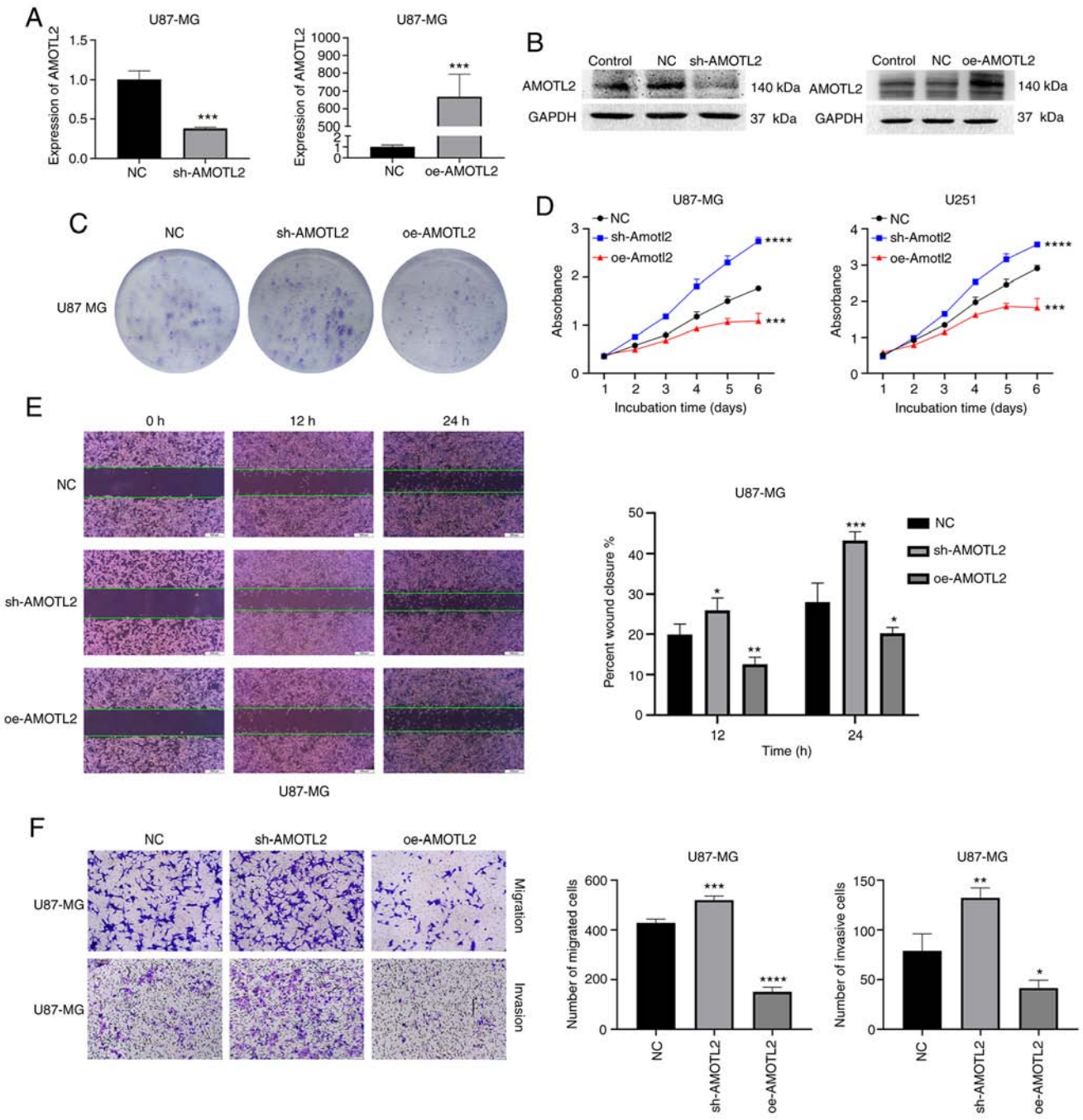

Figure 2. AMOTL2 inhibits the proliferation, migration and invasion of glioma cells. (A and B) The U87MG cell line was transfected with lentiviral vectors of AMOTL2 shRNA and overexpressed plasmids of AMOTL2. Reverse transcription-quantitative PCR and western blotting were performed to examine the AMOTL2 expression level with control and treatment groups. (C) Colony formation assays were conducted using the U87MG cell line to examine the effect of the expression of AMOTL2 in cell colony formation (n=3). (D) The effect of AMOTL2 on proliferation was examined by Cell Counting Kit-8 assay in U87MG and U251 cell lines (n=3). (E) The migrating ability of U87MG cells was detected by wound healing assay (n=5). (F) Transwell migration (upper images) and Matrigel® invasion (lower images) assays were conducted to investigate the effect of AMOTL2 knockdown on U87MG cell migration and invasion (n=5). Results are presented as the mean ± SD. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. AMOTL2, angiomotin-like 2; NC, negative control; sh-, short hairpin; oe, overexpression.

could reverse the AMOTL2-silencing-induced increase in the proliferative ability of glioma.

Subsequently, rescue assays of migration and invasion were conducted. It was shown by the wound healing assay results that XAV939 could suppress the increased wound-healing rate of AMOTL2-silenced cells (Figs. 5D and S2D). The Transwell assays with or without Matrigel® indicated that the AMOTL2-downregulation-induced increase of migration and invasion of glioma were significantly reduced following XAV939 treatment (Figs. 5E and S2E). These results indicated that Wnt pathway inhibitor XAV939 could reverse the AMOTL2-silencing-induced increase in the migratory and invasive abilities of glioma.

In conclusion, these results revealed that the Wnt/β-catenin signaling pathway inhibitor XAV939 could reverse the AMOTL2-knockdown-induced proliferation, migration and

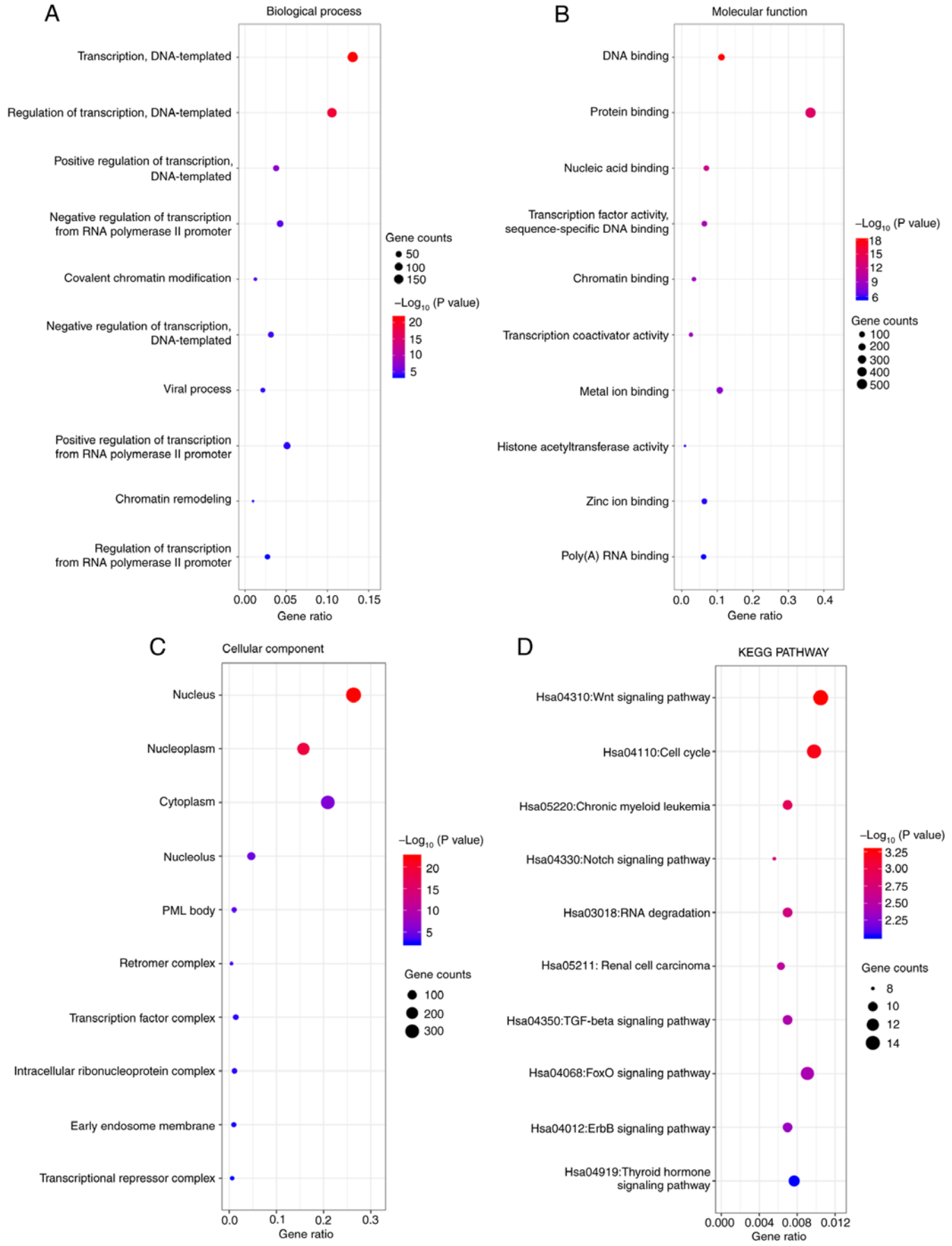

Figure 3. Gene Ontology and KEGG analyses of AMOTL2. The data was downloaded from the Chinese Glioma Genome Atlas database (dataset ID: mRNAseq_693). (A) Biological process, (B) molecular function, (C) cellular component and (D) KEGG pathway analyses of AMOTL2. The bubble diagram was constructed using the 'ggplots' R language package. KEGG, Kyoto Encyclopedia of Genes and Genomes; AMOTL2, angiomotin-like 2.

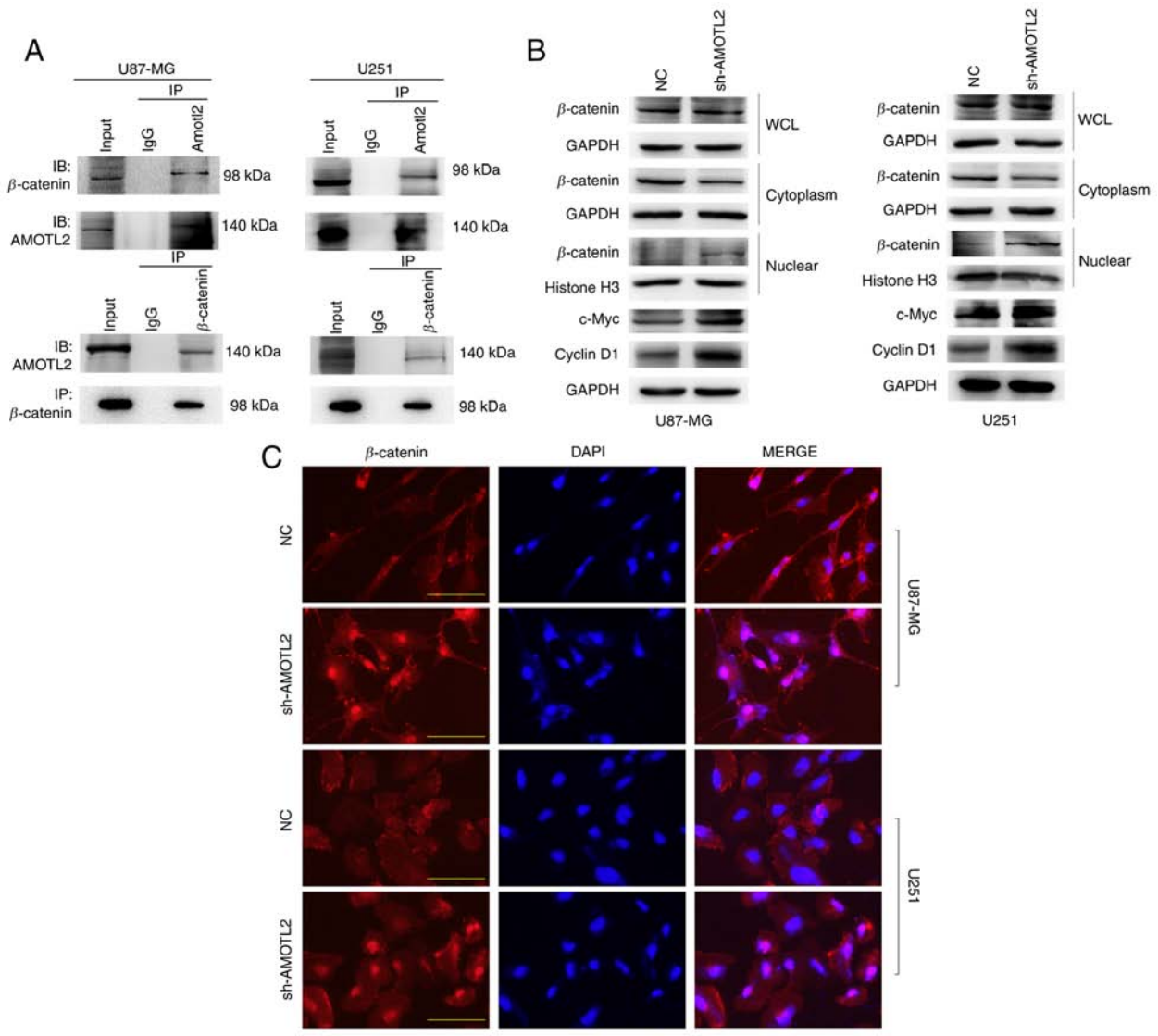

Figure 4. AMOTL2-knockdown activates the Wnt signaling pathway by promoting β-catenin nuclear translocation. (A) Extracts of U87MG and U251 cells were lysed in IP lysis buffer, incubated with AMOTL2 antibody or control IgG, immunoblotted with β-catenin antibody and AMOTL2 antibody (upper images). Reciprocal IP was performed using β-catenin antibody or control IgG, and immunoblotted with AMOTL2 antibody and β-catenin antibody (lower images). (B) Western blotting examined the expression of β-catenin in the whole cell lysate, cytoplasm and nucleus, as well as that of c-Myc and Cyclin D1. (C) Double immunofluorescence staining for β-catenin (red) and nuclear DAPI (blue) was conducted in sh-AMOTL2 or NC U87MG and U251 cells (scale bar, 50 μm). AMOTL2, angiomotin-like 2; IP, immunoprecipitation; NC, negative control; sh-, short hairpin; WCL, whole cell lysate.

invasion of glioma. Collectively, AMOTL2 could regulate the proliferation, migration and invasion of glioma through the Wnt/β-catenin signaling pathway.

Discussion

AMOTL2 is a member of the motin family of angiostatin-binding proteins. During the formation of new blood vessels, angiostatin can inhibit the tube formation and migration of endothelial cells toward growth factors, while AMOTL2 can regulate this effect to maintain normal physiological functions (7). Previous studies have reported that AMOTL2 is closely associated with the occurrence and progression of cancer. However, these studies mainly focused on breast, lung, pancreatic, liver and cervical cancer (10,27-29). Research on the relationship between AMOTL2 and glioma is limited. Similarly, research that focused on the mechanism of AMOTL2 in cancer, on the regulation of YAP, AKT protein and Hippo signaling pathways was also limited (10,30,31). In the present study, a novel mechanism through which AMOTL2 regulates the development of glioma was reported.

Expression and survival analyses of glioma were performed using data from TGGA and CGGA databases. The results indicated that the expression of AMOTL2 was reduced in GBM, as compared with low-grade glioma, and the patients with a high AMOTL2 expression had a longer survival. IHC of clinical glioma specimens yielded the same result. The data from the phenotype experiment demonstrated that AMOTL2 inhibited the tumor proliferation, migration and invasion of glioma. To further explore the mechanism of AMOTL2 in glioma, GO and KEGG analyses were conducted using data from the CGGA. The results revealed that AMOTL2 performed its function by 'protein binding', and the pathway AMOTL2 was significantly enriched in was the Wnt/β-catenin signaling pathway.

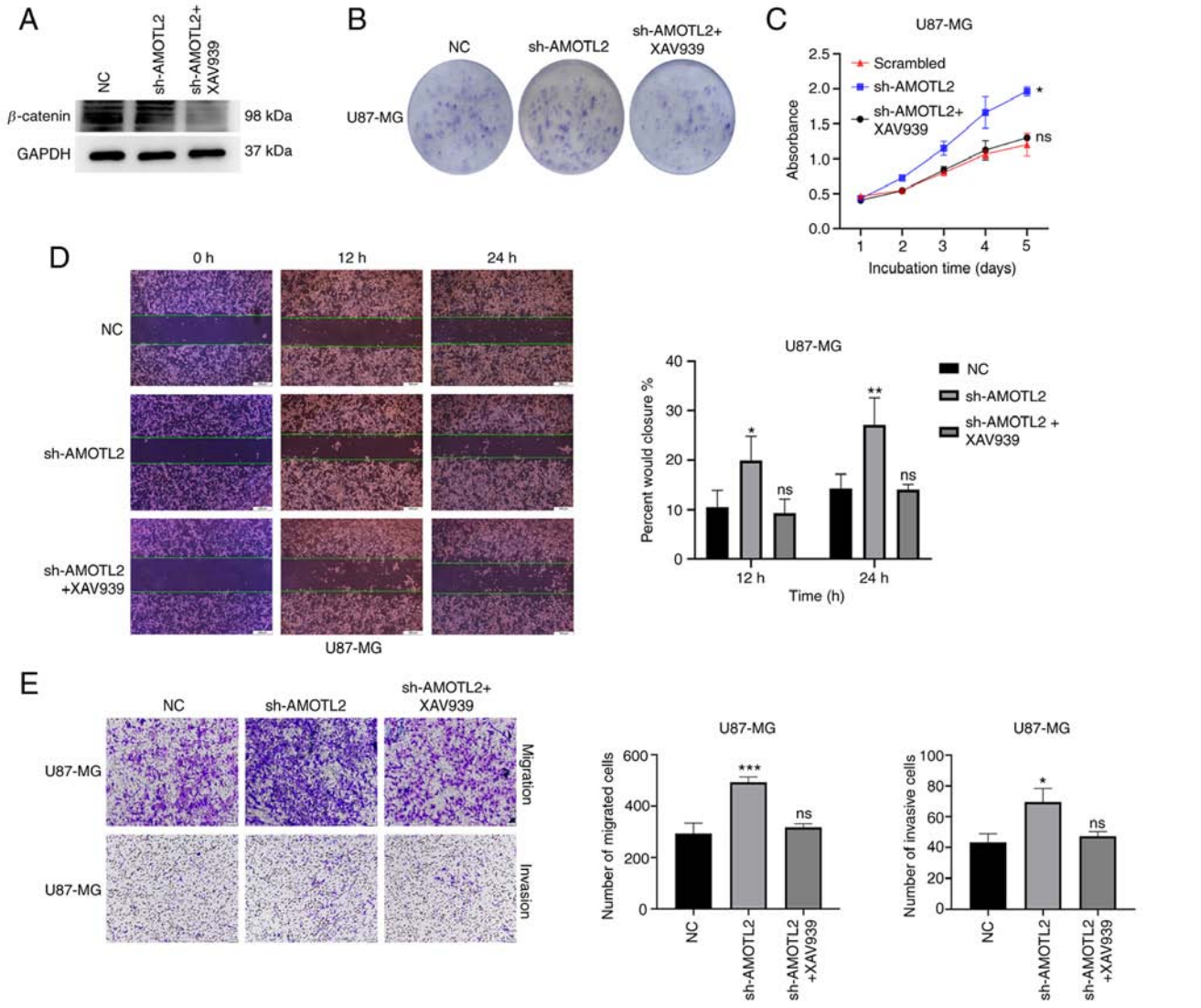

Figure 5. AMOTL2-knockdown can promote the proliferation, migration and invasion of glioma through the Wnt/β-catenin signaling pathway. (A) U87MG cells transfected with lentiviral vectors of AMOTL2 shRNA were treated with Wnt pathway inhibitor XAV939 for 24 h. Western blotting was performed to examine the effect of the inhibition. (B) Colony formation assay was conducted on the U87MG cell line (n=3). (C) Cell Counting Kit-8 assay was conducted to detect the growth of U87MG cells at 1, 2, 3, 4 and 5 days (n=3). (D) The migrating ability of U87MG cells was detected by wound healing assay (n=5). (E) Transwell migration (upper images) and Matrigel® invasion (lower images) assays were conducted to examine migration and invasion (n=5). Results are presented as the mean ± SD. *P<0.05, **P<0.01 and ***P<0.001. AMOTL2, angiomotin-like 2; ns, not significant; sh-, short hairpin; NC, negative control.

The Wnt/β-catenin signaling pathway is one of the classic pathways that can affect several physiological processes, such as cell proliferation, differentiation, organogenesis, tissue regeneration and tumorigenesis (12,32-35). Several studies have revealed that the aberrant regulation of the Wnt/β-catenin signaling pathway occurs frequently in glioma (36,37). Therefore, several studies were performed to examine the potential role of the Wnt signaling pathway as a novel therapeutic target for glioma. In this context, it appears using aberrant Wnt/β-catenin signaling as a therapeutic target is a lucrative approach to combat cancer (38). Treating cancer through the suppression of the Wnt signaling is an emerging therapeutic method, and several inhibitors of the Wnt pathway, such as PORCN inhibitors, Wnt ligand antagonists and FZD antagonists/monoclonal antibodies (39-41) have been used in various cancer-related clinical trials. However, due to the positive regulatory function of Wnt/β-catenin signaling over biological processes, including the maintenance of stem cell pool stability and regeneration of tissues and organs (42), the interdiction of Wnt signaling may cause irreversible damage to physiological homeostasis and regenerative ability (43). Therefore, to further use these molecules in the clinical setting, an understanding of their potential mechanism needs to be obtained through additional preclinical and clinical work.

To demonstrate whether AMOTL2 can directly interact with β-catenin to regulate the Wnt pathway, Co-IP assay and western blotting were performed. It was revealed that AMOTL2 could bind to the β-catenin protein and the knockdown of AMOTL2 could activate downstream genes of the Wnt pathway, such as c-Myc and cyclin D1. Nevertheless, the expression level of β-catenin protein was not altered when AMOTL2 was silenced.

It was reported that, in the canonical Wnt pathway, when the Wnt signal was in an inactivated state, the β-catenin destruction complex, which contains the scaffold protein Axin, APC and the kinases GSK3β and casein kinase, would be activated, leading to β-catenin protein phosphorylatation by

the destruction complex, and degradation by proteasomes (12). Conversely, when Wnt signaling is activated, β-catenin proteins tend to be stable and are constantly accumulated, finally translocating into the nucleus. In the nucleus, β-catenin forms an active complex with T-cell factor and lymphoid enhancer factor proteins by displacing the TLE/Groucho complexes and recruiting histone modifying co-activators, such as CBP/p300, BRG1, BCL9 and Pygo (44). This transcriptional switch leads to changes in multiple cellular processes (45,46).

Therefore, it was hypothesized that the role of AMOTL2 in regulating Wnt pathway genes was not directly played through the β-catenin destruction complex, but rather by controlling its nuclear translocation. To verify this hypothesis, nuclear and cytoplasmic proteins of the control and knockdown groups, respectively, were extracted to conduct western blotting. Next, immunofluorescence staining was performed to locate the distribution of β-catenin in cells. The results revealed that the downregulation of AMOTL2 promoted the nuclear translocation of β-catenin. These results indicated that AMOTL2 could keep β-catenin from translocating into the nucleus by directly binding to β-catenin protein.

To further demonstrate that AMOTL2 regulates the proliferative, migrating and invasive abilities of glioma cells through the Wnt/β-catenin signaling pathway, the Wnt/β-catenin pathway inhibitor XAV939 was used to perform the phenotype rescue assays. The results indicated that the Wnt/β-catenin pathway inhibitor XAV939 could reverse the AMOTL2-silencing-induced proliferation, migration and invasion of glioma. It was reported that AMOTL2 could regulate the development of glioma cells through the Wnt/β-catenin pathway.

In conclusion, the present study revealed that AMOTL2 could regulate the proliferation, migration and invasion of glioma cells by directly binding to the β-catenin protein to control the nuclear translocation of β-catenin. Therefore, AMOTL2 may serve as a novel therapeutic target in glioma treatment. Furthermore, based on the association between AMOTL2 and β-catenin, AMOTL2 may contribute to the study of targeting therapeutics of Wnt signaling. In view of the several side effects of Wnt signaling inhibitors, further research should be performed on the interaction between AMOTL2 and β-catenin. In addition, there are some limitations in the clinical transformation of this study. The present study only demonstrated the effect of AMOLT2 on glioma through the Wnt/β-catenin pathway at the cellular level, but lacks verification at the animal level. Moreover, the application of AMOTL2 as an inhibitor of the Wnt pathway is only at a preliminary stage, and more basic and clinical validation is required. Therefore, additional studies are necessary to understand the potential mechanism and clinical value of AMOTL2.

Acknowledgements

Not applicable.

Funding

This work was supported by the National Natural Science Foundation of China (grant nos. 81172405 and 81572490), the Tianjin Science and Technology Committee (grant no. 18JCZDJC98600) and the Science and Technology fund of Tianjin Binhai New Area Health and Family Planning Commission (grant no. 2018BWKZ003).

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

XC and YL performed the experiments and compiled the manuscript. GG contributed to the design of the study and analyzed data. YZ and YS conducted experiments and analyzed data. LG, RL and YN acquired and interpreted the data. XY, JD and XJ collected clinical specimens and analyzed the data. QH contributed to the conception and design of the present study and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All clinical specimen tissues were reviewed and approved by the Ethics Committee of Tianjin Medical University General Hospital (Tianjin, China), and informed consents were signed by the patients according to the Declaration of Helsinki. (approval no. IRB2021-WZ-006).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Anton K, Baehring JM and Mayer T: Glioblastoma multiforme: Overview of current treatment and future perspectives. Hematol Oncol Clin North Am 26: 825-853, 2012.
2. Ostrom QT, Gittleman H, Liao P, Vecchione-Koval T, Wolinsky Y, Kruchko C and Barnholtz-Sloan JS: CBTRUS Statistical Report: Primary brain and other central nervous system tumors diagnosed in the United States in 2010-2014. Neuro Oncol 19 (Suppl 5): v1-v88, 2017.
3. Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, Ohgaki H, Wiestler OD, Kleihues P and Ellison DW: The 2016 World Health Organization classification of tumors of the central nervous system: A summary. Acta Neuropathol 131: 803-820, 2016.
4. Omuro A and DeAngelis LM: Glioblastoma and other malignant gliomas: A clinical review. JAMA 310: 1842-1850, 2013.
5. Lv M, Shen Y, Yang J, Li S, Wang B, Chen Z, Li P, Liu P and Yang J: Angiomotin family members: Oncogenes or tumor suppressors? Int J Biol Sci 13: 772-781, 2017.
6. Huang T, Zhou Y, Zhang J, Cheng ASL, Yu J, To KF and Kang W: The physiological role of Motin family and its dysregulation in tumorigenesis. J Transl Med 16: 98, 2018.
7. Lv M, Li S, Luo C, Zhang X, Shen Y, Sui YX, Wang F, Wang X, Yang J, Liu P and Yang J: Angiomotin promotes renal epithelial and carcinoma cell proliferation by retaining the nuclear YAP. Oncotarget 7: 12393-12403, 2016.
8. Moreau J, Lord M, Boucher M, Belleau P and Fernandes MJG: Protein diversity is generated within the motin family of proteins by alternative pre-mRNA splicing. Gene 350: 137-148, 2005.
9. Kim M, Kim M, Park S-J, Lee C and Lim DS: Role of angiomotin-like 2 mono-ubiquitination on YAP inhibition. EMBO Rep 17: 64-78, 2016.

10. Han H, Yang B and Wang W: Angiomotin-like 2 interacts with and negatively regulates AKT. Oncogene 36: 4662-4669, 2017.
11. Nusse R and Clevers H: Wnt/β-catenin signaling, disease, and emerging therapeutic modalities. Cell 169: 985-999, 2017.
12. Zhan T, Rindtorff N and Boutros M: Wnt signaling in cancer. Oncogene 36: 1461-1473, 2017.
13. Vallée A and Lecarpentier Y: Crosstalk between peroxisome proliferator-activated receptor gamma and the canonical WNT/β-catenin pathway in chronic inflammation and oxidative stress during carcinogenesis. Front Immunol 9: 745, 2018.
14. He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B and Kinzler KW: Identification of c-MYC as a target of the APC pathway. Science 281: 1509-1512, 1998.
15. Brabletz T, Jung A, Dag S, Hlubek F and Kirchner T: Beta-catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer. Am J Pathol 155: 1033-1038, 1999.
16. ten Berge D, Koole W, Fuerer C, Fish M, Eroglu E and Nusse R: Wnt signaling mediates self-organization and axis formation in embryoid bodies. Cell Stem Cell 3: 508-518, 2008.
17. Blache P, van de Wetering M, Duluc I, Domon C, Berta P, Freund JN, Clevers H and Jay P: SOX9 is an intestine crypt transcription factor, is regulated by the Wnt pathway, and represses the CDX2 and MUC2 genes. J Cell Biol 166: 37-47, 2004.
18. Clevers H and Nusse R: Wnt/β-catenin signaling and disease. Cell 149: 1192-1205, 2012.
19. Espada J, Calvo MB, Díaz-Prado S and Medina V: Wnt signalling and cancer stem cells. Clin Transl Oncol 11: 411-427, 2009.
20. Wang Y, Qian T, You G, Peng X, Chen C, You Y, Yao K, Wu C, Ma J, Sha Z, *et al*: Localizing seizure-susceptible brain regions associated with low-grade gliomas using voxel-based lesion-symptom mapping. Neuro Oncol 17: 282-288, 2015.
21. Liu X, Li Y, Qian Z, Sun Z, Xu K, Wang K, Liu S, Fan X, Li S, Zhang Z, *et al*: A radiomic signature as a non-invasive predictor of progression-free survival in patients with lower-grade gliomas. Neuroimage Clin 20: 1070-1077, 2018.
22. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, *et al*: Gene ontology: Tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 25: 25-29, 2000.
23. Kanehisa M: The KEGG database. Novartis Found Symp 247: 91-103, 119-128, 244-252, 2002.
24. Huang DW, Sherman BT, Tan Q, Collins JR, Alvord WG, Roayaei J, Stephens R, Baseler MW, Lane HC and Lempicki RA: The DAVID Gene functional classification tool: A novel biological module-centric algorithm to functionally analyze large gene lists. Genome Biol 8: R183, 2007.
25. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
26. Krieghoff E, Behrens J and Mayr B: Nucleo-cytoplasmic distribution of beta-catenin is regulated by retention. J Cell Sci 119: 1453-1463, 2006.
27. Jiang WG, Watkins G, Douglas-Jones A, Holmgren L and Mansel RE: Angiomotin and angiomotin like proteins, their expression and correlation with angiogenesis and clinical outcome in human breast cancer. BMC Cancer 6: 16, 2006.
28. Cui R, Jiang N, Zhang M, Du S, Ou H, Ge R, Ma D and Zhang J: AMOTL2 inhibits JUN Thr239 dephosphorylation by binding PPP2R2A to suppress the proliferation in non-small cell lung cancer cells. Biochim Biophys Acta Mol Cell Res 1868: 118858, 2020.
29. Guo Z, Wang X, Yang Y, Chen W, Zhang K, Teng B, Huang C, Zhao Q and Qiu Z: Hypoxic tumor-derived exosomal long noncoding RNA UCA1 promotes angiogenesis via miR-96-5p/AMOTL2 in pancreatic cancer. Mol Ther Nucleic Acids 22: 179-195, 2020.
30. Hong W: Angiomotin'g YAP into the nucleus for cell proliferation and cancer development. Sci Signal 6: pe27, 2013.
31. Zhao B, Li L, Lu Q, Wang LH, Liu CY, Lei Q and Guan KL: Angiomotin is a novel Hippo pathway component that inhibits YAP oncoprotein. Genes Dev 25: 51-63, 2011.
32. Acebron SP, Karaulanov E, Berger BS, Huang YL and Niehrs C: Mitotic Wnt signaling promotes protein stabilization and regulates cell size. Mol Cell 54: 663-674, 2014.
33. Atlasi Y, Noori R, Gaspar C, Franken P, Sacchetti A, Rafati H, Mahmoudi T, Decraene C, Calin GA, Merrill BJ and Fodde R: Wnt signaling regulates the lineage differentiation potential of mouse embryonic stem cells through Tcf3 down-regulation. PLoS Genet 9: e1003424, 2013.
34. Clevers H, Loh KM and Nusse R: Stem cell signaling. An integral program for tissue renewal and regeneration: Wnt signaling and stem cell control. Science 346: 1248012, 2014.
35. Green JL, Inoue T and Sternberg PW: Opposing Wnt pathways orient cell polarity during organogenesis. Cell 134: 646-656, 2008.
36. Kristensen BW, Priesterbach-Ackley LP, Petersen JK and Wesseling P: Molecular pathology of tumors of the central nervous system. Ann Oncol 30: 1265-1278, 2019.
37. He L, Zhou H, Zeng Z, Yao H, Jiang W and Qu H: Wnt/β-catenin signaling cascade: A promising target for glioma therapy. J Cell Physiol 234: 2217-2228, 2019.
38. Jung YS and Park JI: Wnt signaling in cancer: Therapeutic targeting of Wnt signaling beyond β-catenin and the destruction complex. Exp Mol Med 52: 183-191, 2020.
39. Madan B, Ke Z, Harmston N, Ho SY, Frois AO, Alam J, Jeyaraj DA, Pendharkar V, Ghosh K, Virshup IH, *et al*: Wnt addiction of genetically defined cancers reversed by PORCN inhibition. Oncogene 35: 2197-2207, 2016.
40. Giraudet AL, Cassier PA, Iwao-Fukukawa C, Garin G, Badel JN, Kryza D, Chabaud S, Gilles-Afchain L, Clapisson G, Desuzinges C, *et al*: A first-in-human study investigating biodistribution, safety and recommended dose of a new radiolabeled MAb targeting FZD10 in metastatic synovial sarcoma patients. BMC Cancer 18: 646, 2018.
41. Jimeno A, Gordon M, Chugh R, Messersmith W, Mendelson D, Dupont J, Stagg R, Kapoun AM, Xu L, Uttamsingh S, *et al*: A first-in-human phase I study of the anticancer stem cell agent ipafricept (OMP-54F28), a decoy receptor for Wnt ligands, in patients with advanced solid tumors. Clin Cancer Res 23: 7490-7497, 2017.
42. Patel S, Alam A, Pant R and Chattopadhyay S: Wnt signaling and its significance within the tumor microenvironment: Novel therapeutic insights. Front Immunol 10: 2872, 2019.
43. Galluzzi L, Spranger S, Fuchs E and López-Soto A: WNT signaling in cancer immunosurveillance. Trends Cell Biol 29: 44-65, 2019.
44. Lien WH and Fuchs E: Wnt some lose some: Transcriptional governance of stem cells by Wnt/β-catenin signaling. Genes Dev 28: 1517-1532, 2014.
45. Clevers H: Wnt/beta-catenin signaling in development and disease. Cell 127: 469-480, 2006.
46. MacDonald BT, Tamai K and He X: Wnt/beta-catenin signaling: Components, mechanisms, and diseases. Dev Cell 17: 9-26, 2009.