Original Article MicroRNA-206 attenuates glioma cell proliferation, migration, and invasion by blocking the WNT/β-catenin pathway via direct targeting of Frizzled 7 mRNA

Fengqi Zhou^{*}, Wenping Cao^{*}, Ran Xu^{*}, Junxia Zhang, Tianfu Yu, Xiupeng Xu, Tongle Zhi, Jianxing Yin, Shengwu Cao, Ning Liu, Yingyi Wang, Chunsheng Zhao

Department of Neurosurgery, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, Jiangsu Province, China. *Equal contributors.

Received February 23, 2019; Accepted June 24, 2019; Epub July 15, 2019; Published July 30, 2019

Abstract: Glioma is one of the most prevalent primary malignant brain tumours among adults, and accumulating evidence has shown that dysregulation of microRNAs (miRNAs) is associated with various types of cancers, including glioma. It is necessary to gain a better understanding of the roles and mechanisms of action of miRNAs in WNT-driven glioblastoma multiforme (GBM). Here, we report that miR-206 inhibits the WNT/β-catenin pathway by directly targeting Frizzled 7 (*FZD7*) mRNA and functions as a tumour suppressor in glioma. The expression of miR-206 in human glioma samples and glioma cells was assessed by reverse-transcription quantitative PCR, fluorescence *in situ* hybridisation, and histological analysis. Cell Counting Kit-8, colony formation, 5-ethynyl-2'-deoxyuridine incorporation, flow-cytometric, wound healing, Transwell invasion, and three-dimensional migration assays were performed to examine glioma cell proliferation, migration, and invasion *in vitro*. The effects of miR-206 *in vivo* were investigated in a xenograft nude-mouse model. MiR-206 expression was significantly lower in glioma specimens than in normal control samples. *FZD7* was confirmed as a direct target gene of miR-206. GBM cell proliferation, migration, and invasion were blocked after restoration of miR-206 expression. Moreover, intracranial glioma models revealed an inhibitory effect of miR-206 on intracranial glioma tumour growth. Our results suggest that miR-206 plays a key role in the blockade of the WNT/β-catenin signalling pathway by down-regulating FZD7 and may be a promising therapeutic agent against malignant glioma and other WNT-driven tumours.

Keywords: miR-206, glioma, FZD7, proliferation, invasion

Introduction

Glioma, one of the most prevalent primary malignant tumours of the nervous system in adults, arises in glial tissue [1]. Gliomas have a fatal prognosis in most patients and significantly affect the physical and mental health of the patients [2]. Glioblastoma multiforme (GBM), the most malignant form of glioma, is one of the deadliest human cancers [3]. Although there have been improvements in standard therapeutic strategies, glioma treatment remains inadequate, with a median survival time of only 15 months for patients with GBM [4, 5]. Thus, the molecular mechanisms of glioma progression should be further explored and novel therapies-that can improve patient outcomes-should be developed.

MicroRNAs (miRNAs) are short non-coding RNA molecules that perform key functions in the progression of GBM. They bind to the 3' untranslated region (3'-UTR) of their target messenger RNA (mRNA) to down-regulate gene expression [6, 7]. MiRNAs probably regulate approximately 30% of all human oncogenes and tumour suppressors [8, 9]. In recent years, accumulating evidence revealed that miRNA dysregulation affects a variety of cellular phenomena, such as proliferation, cell cycle progression, invasion, migration, apoptosis, metabolism, differentiation, and stress responses [10]. In particular, miR-206 is down-regulated in multiple tumour types, such as hepatocellular carcinoma, osteosarcoma, prostate cancer, breast cancer, and cervical cancer [11-15]. Our preliminary experiments showed that miR-206 is down-regulated

in glioma and that miR-206 expression significantly negatively correlates with histopathological grade. However, the exact involvement of miR-206 in GBM is unclear.

The WNT/ β -catenin signalling pathway is a highly conserved cascade that has been reported to participate in malignant progression and is significantly associated with the poor prognosis of malignant tumours by modulating multiple parameters of tumour initiation, growth, and metastasis [16]. It has also been demonstrated that the WNT/β-catenin signalling pathway plays a vital part in the regulation of glioma fate determination [17]. When the seven-pass transmembrane receptor called Frizzled binds to its ligand (WNT), the WNT/ β -catenin signalling pathway is activated and unphosphorylated β-catenin enters the nucleus. There, it forms multimeric complexes by binding to a transcription factor (T-cell factor or lymphoid-enhancing factor (TCF or LEF, respectively) to regulate the expression of multiple downstream β-catenindependent genes involved in various cellular functions, e.g. c-Myc, cyclin D1, CD44, and matrix metalloproteinases (MMPs) [18, 19]. Human frizzled 7 (FZD7), which is located in chromosomal region 8q22.3-q23.1 [20], is expressed in various tissues, including skeletal muscle, the heart, brain, placenta, kidneys, and lungs [21]. Lately, an increasing number of studies has been showing that FZD7 activates the WNT/β-catenin signalling pathway in several cancers, such as hepatocellular carcinoma, co-Ion cancer, gastric cancer, and triple-negative breast cancer [22-26]. WNT3 serves as a ligand of FZD7. It was recently discovered that FZD7 acts as an oncogene.

In the present study, miR-206 expression was found to be significantly inversely associated with the World Health Organization (WHO) tumour grade and was lower in clinical GBM specimens than in non-cancerous brain tissue (NBT) samples. Furthermore, we provided evidence that miR-206 regulates the progression of glioma by directly targeting *FZD7* mRNA thereby blocking the WNT/ β -catenin signalling pathway. Generally speaking, the miR-206/FZD7/WNT axis plays an important role in the progression of GBM; therefore, a greater understanding of this axis may point to a novel clinically effective therapy for malignant glioma.

Materials and methods

Human tissue samples

Thirty-three human glioma samples were obtained post-operatively from the Department of Neurosurgery at the First Affiliated Hospital of Nanjing Medical University (Nanjing, China). In addition, six NBT samples were collected as negative controls from patients who underwent decompressive craniotomy for severe traumatic brain injury. All the resected tissue samples were frozen in liquid nitrogen immediately. Both glioma and NBT samples were confirmed histologically. Our study protocol was approved by the Research Ethics Committee of Nanjing Medical University (Nanjing, Jiangsu, China), and written informed consent was obtained from each enrolled patient. Public datasets are described in the Supplementary Materials and in the Methods section.

Public datasets

A total of 158 gliomas with microarray miRNA expression data were downloaded from the Chinese Glioma Genome Atlas (CGGA) data portal (http://www.cgga.org.cn/portal.phpg). Gene expression data were downloaded from The Cancer Genome Atlas (TCGA) database (http://tcga-data.nci.nih.gov/) and the GSE4-290 (https://www.ncbi.nlm.nih.gov/geo/query/ acc.cgi?acc=GSE4290) and GSE16011 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi? acc=+GSE16011) data sets. Six processed immunohistochemical staining of glioma patients were downloaded from Human Protein Atlas database (http://www.proteinatlas.org/).

Cell culture, transfection, and establishment of stably virus-transduced cell lines

Six human glioblastoma (GBM) cell lines (U87, LN229, U251, T98G, A172, and U118) were purchased from the Chinese Cell Repository (Shanghai, China). Normal human astrocytes (NHAs) were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA). All cells were grown at 37°C in a 5% CO_2 incubator. Small interfering (si) FZD7 and control si-non-coding (siNC) oligonucleotides were purchased from GenePharma (Shanghai, China). The FZD7-targeting sequence of the siRNA was 5'-GCACC-

Table 1. Primer sequences f	or RT-qPCR
-----------------------------	------------

Gene	Primer sequence (5'-3')
miR-206	Forward: 5'-GCGTCTGGAATGTAAGGAAGTG-3'
	Reverse: 5'-GTGCAGGGTCCGAGGT-3'
FZD7	Forward: 5'-TTCGTCCCTGGGCCTCT-3'
	Reverse: 5'-TGGTCTGGTTGTAGGCGATG-3'
GAPDH	Forward: 5'-GAGTCAACGGATTTGGTCGT-3'
	Reverse: 5'-TTGATTTTGGAGGGATCTCG-3'
U6	Forward: 5'-TTGGTCTGATCTGGCACATATAC-3'
	Reverse: 5'-AAAAATATGGAGCGCTTCACG-3'

Note: miR-206, microRNA-206; FZD7, Frizzled7; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

ATCATGAACACGACG-3' and 5'-ACCTGATGACC-ATGATCGTC-3'. An FZD7-overexpression plasmid was generated by inserting the FZD7 coding region into a pcDNA3.1 vector. An HasmiR-206 mimic and has-miR-ctrl were obtained from RiboBio (Guangzhou, China). All oligonucleotides and plasmids were transfected using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA). Lentivirus vectors with has-miR-206 or has-miR-control (miRctrl) were purchased from Genechem (Shanghai, China). Lentiviruses were packaged in the human embryonic kidney cell line, 293T, and virions were collected according to the manufacturer's instructions. Stable U87 and LN229 cell lines were established by lentiviral infection and puromycin selection following the manufacturer's instructions.

RNA extraction and reverse-transcription quantitative PCR (RT-qPCR)

Total RNA was extracted from harvested cells or human tissues with TRIzol reagent according to the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA). The stem-loopspecific primer method was used to quantify the expression levels of miR-206. miRNA-specific reverse transcription primers and quantitative PCR primers were purchased form RiboBio. U6 small nuclear RNA levels served as an internal control. cDNA was amplified by qRT-PCR using SYBR Premix ExTaq (Takara, Kusatsu, Japan) on a 7900HT system (Applied Biosystems, Foster City, CA, USA). Primers for qRT-PCR were purchased from Genepharma. All experiments were performed in triplicate and repeated three times. Fold changes were calculated by relative quantification (2-AACt), See Table 1 for details on primer sequences for RT-qPCR.

Western blotting and antibodies

Proteins were extracted from cells and tissues with RIPA lysis buffer (KeyGEN, Nanjing, China). Equal amounts of protein from each lysate were separated by SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Thermo Fisher Scientific, Waltham, MA, USA). The following primary antibodies were purchased from Abcam (Cambridge, UK) for immunoblotting: anti-FZD7 (cat. #ab64636), anticyclin D1 (ab40754), anti-CDK2 (ab32147), anti-Slug (ab27568), anti-N-cadherin (ab182-03), anti-vimentin (ab92547), anti-β-catenin (ab32572), anti-c-Myc (ab32072), and antitubulin (ab7291). Anti-MMP9 (#13667S) and anti-MMP2 (#40994S) antibodies were acquired from Cell Signaling Technology (Danvers, MA, USA).

Cell proliferation assays

After transfection, cell growth was analysed by Cell Counting Kit-8 (Dojindo, Kumamoto, Japan), according to the manufacturers protocol. For the colony formation assay, stably transfected U87 and LN229 cells were independently seeded onto a six-well plate (200 cells per well). Cells were incubated for approximately 2 weeks at 37°C in a 5% CO₂ incubator. After colony formation was observed, colonies were fixed with 4% paraformaldehyde and stained with 1% crystal violet (Sigma, St Louis, MO, USA) for 30 min. A Click-iT EdU Alexa Fluor 594 Imaging Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used following the manufacturer's instructions and samples were imaged under a fluorescence microscope.

Cell cycle analysis

At 48 h post-transfection, cells were collected by centrifugation, washed with phosphate-buffered saline (PBS), and ruptured in 70% ice-cold ethanol overnight. After centrifuging, the supernatant was discarded. Before analysis with a flow cytometer, the cells were washed twice with PBS, resuspended using a Cell Cycle Staining Kit (Multi Sciences, Hangzhou, China), and incubated in the dark for 30 min.

Cell migration and invasion experiments

For the wound healing scratch assay, a uniform wound was made by scratching with a pipette tip (standard 200 μ L) when the stably transfect-

ed U87 and LN229 cells reached 100% confluence in 6-well plates. Cells were maintained in serum-free DMEM after been washed thrice with PBS. After 24 h, each well was photographed under an inverted microscope (Nikon, Tokyo, Japan) and the cells protruding from the border of the scratches were counted.

To test cell invasion, plates with transwell inserts (Corning, New York, NY, USA) that were pre-coated with 20 µg/µL of Matrigel (BD Biosciences, San Jose, CA, USA) were used. Stably transfected U87 and LN229 cells at a density of 3×10⁴ cells/ml in serum-free DMEM were transferred to the upper chambers. In parallel, DMEM containing 10% FBS was added to the lower chamber of each well. After incubation for 24 h at 37°C and removal of non-invading cells, the invading cells were fixed with 4% paraformaldehyde for 15 min and then stained with 0.1% crystal violet for 30 min. Six fields of invading cells in each well were captured and counted randomly under a microscope at ×100 magnification.

For 3D spheroid assays, transfected cells were grown to 70% confluence and were then seeded in 96-well ultra-low adherence plates (#7007, Costar) at a density of 0.2×10^5 cells/ mL. After 96 h, these cells were induced to aggregate into a multicellular spheroid and then Matrigel was added into each well. At 48 h, fluorescence microscopy was used to analyse cell motion.

Fluorescent staining

Cells fixed by 4% formalin were incubated with rabbit monoclonal anti-FZD7 (Abcam Cambridge, UK, cat. #ab64636) and mouse monoclonal anti- β -catenin (Abcam Cambridge, UK, cat. #2677) overnight at 4°C and then with Alexa 488- or 568-labeled anti-mouse or anti-rabbit IgG antibody (Thermo Fisher Scientific, Massachusetts, USA) 2 h at room temperature. After treated with DAPI (Beyotime Biotechnology, Jiangsu, China) for 10 min, cells were examined with Zeiss axiophot photomicroscope (Carl Zeiss AG, Jena, Germany).

A dual-luciferase reporter assay

For reporter assays, U87 and LN229 cells were seeded in 24-well plates and transfected (using Lipofectamine 2000) with wild-type or mutated plasmid pGL3-FZD7, together with pRL-SV40 (Promega, Madison, WI, USA) expressing *Renilla* luciferase and an miR-206 mimic (RiboBio,

Guangzhou, China). After 48 h of incubation, the cells were harvested, and luciferase activity was measured using the Dual-Luciferase Reporter Assay Kit (Promega).

Immunohistochemistry

Fresh mouse brain tissue was fixed in 4% paraformaldehyde, embedded in paraffin, and fivemicron-thick sections were prepared. Sections were stained with Mayr's haematoxylin and subsequently with eosin (Biogenex Laboratories, Fremont, CA, USA) or an antibody against FZD7, MMP2, or Ki-67. Brown staining in cells was considered as positive signal.

A murine intracranial glioma model and immunohistochemical (IHC) analysis

Eighteen male Bagg albino (BALB)/c nude mice (4 weeks old) were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences. U87 cells stably expressing luciferase were transfected with lentiviral vector LV-miR-206 or LV-miR-Ctrl (expressing miR-206 or negative control miRNA, respectively), and then mice were subdivided randomly into an miR-206 over-expression group and control group. To establish the intracranial GBM model, each mouse was implanted stereotactically with U87^{luciferase+miR-206} or U87^{luciferase+miR-ctrl} cells (5×10⁵) in each group. Intracranial tumour growth was measured by bioluminescence imaging. Each mouse was anesthetised and then given an intraperitoneal injection of p-luciferin (50 mg/mL). Tumours were imaged by means of the Living Image software package (Caliper Life Science, Waltham, MA, USA). Mouse survival time was monitored until the final mouse died. IHC analysis was performed to quantify proteins FZD7, MMP2, and Ki-67 in each brain tissue section. All the animal experiments were approved in accordance with the Animal Use Guidelines of the Chinese Ministry of Health (documentation 55,2001).

Fluorescence in situ hybridisation (FISH)

FISH was used to detect the expression of miR-206 in GBM and NBT samples. The mature human miR-206 sequence is 3'-UGGAAUGU-AAGGAAGUGUGUGG-5'. Locked nucleic acid (LNA)-based probes directed against the fulllength mature miRNA sequence were used. The 5'-FAM-labelled miR-206 probe sequence was: 5'-ACCTTACATTCCTTCACACACC-3' and was obtained from BioSense (Guangzhou, China). The



Figure 1. MiR-206 is under-expressed in glioma tissue and glioma cell lines. A. Relative expression of miR-206, according to the CGGA database (low-grade [LGG] vs. high-grade [HGG] gliomas). B. The expression of miR-206 was analysed in normal human astrocytes (NHAs) and six glioma cell lines (U251, T98G, A172, U118, U87, and LN229). C. Expression levels of miR-206 in six non-cancerous brain tissue (NBT) samples and eight tissue samples of grade II-IV glioma. D. Relative expression of miR-206 in NBT and GBM samples was assessed by FISH (scale bars are 50 μ m). #P < 0.0001.

FISH procedure was performed according to the instructions provided by BioSense. Briefly, fresh tissues were fixed in 4% formaldehyde for 1 h and then dehydrated in 15% sucrose for 8 h. Tissues were fixed in 4% formaldehyde for 10 min, washed in PBS (pH 7.4) three times for 5 min each, digested with proteinase K for 2 min, and then washed with PBS again three times for another 5 min each. After eliminating autofluorescence and blocking endogenous biotin, the sections were hybridized with probes overnight in a humid chamber. The next day, tissues sections were washed with warmed 2×SSC, 1×SSC, and 0.5×SSC at 37°C for 10 min each in sequence. After incubation in BSA for 30 min at room temperature, tissue sections were treated with Alexa Fluor 488-avidin (1:400), incubated at room temperature for 50 min, and washed three times for 5 min each using PBS. Tissue sections were incubated with primary antibody overnight and then the species-specific secondary antibody for 50 min. Sections were then stained with DAPI (Sigma) for 10 min and examined under an LSM 700 Meta confocal microscope (Zeiss, Oberkochen, Germany).

Statistical analysis

All the experiments were conducted in triplicate. All statistical analyses and Kaplan-Meier survival analysis were performed in GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA). The correlation between miR-206 expression and FZD7 levels in glioma tissues was evaluated by Pearson's correlation analysis. Two-tailed Student's *t* test was performed for other comparisons, and the results are expressed as the mean \pm standard deviation. Data with *P* < 0.05 were considered statistically significant.

Results

MiR-206 is down-regulated in glioma

To investigate the role of miR-206 in the progression of GBM, Chinese Glioma Genome Atlas (CGGA) data were studied to determine the expression of miR-206 in 158 glioma tissue samples. The results indicated that miR-206 was significantly down-regulated in high-grade gliomas (HGGs) as compared with that in lowgrade gliomas (LGGs; Figure 1A). Compared to that in normal human astrocytes, miR-206 expression was lower in GBM cell lines (Figure **1B**). We then carried out RT-qPCR to evaluate miR-206 expression in 33 glioma samples and six NBT samples. Compared to that in NBT samples, miR-206 expression was significantly lower in glioma tissues, especially in HGGs (Figure 1C). Furthermore, by FISH analysis, we uncovered significantly higher miR-206 expression in GBM tissue samples than in NBT samples (Figure 1D). Overall, these results led to the conclusion that miR-206 expression is low in glioma.

Over-expression of miR-206 attenuates GBM cell proliferation

To determine the participation of miR-206 in the progression of glioma, we transfected the GBM cell lines, U87 and LN229, with miR-ctrlor miR-206-expressing lentiviral vectors. After transfection, RT-qPCR was carried out to confirm that miR-206 expression was significantly increased as compared with that in the negative control group (Figure 2A). After confirmation of the successful transfection, proliferation of the transfected GBM cells was measured in vitro. CCK-8 and colony formation assays revealed that over-expression of miR-206 reduced the proliferation rate of (and colony formation by) the lentiviral-vector-transduced U87 and LN229 cells (Figure 2B and 2C). Consistently with the results of the CCK-8 and colony formation assays, the EdU assay indicated that the percentage of EdU-positive U87 and LN229 cells dramatically decreased with miR-206 upregulation (Figure 2D). Because GBM cell growth is often associated with cell cycle dysfunction, we next studied cell cycle features of the cells with abnormal miR-206 expression. Flow-cytometric analysis was conducted to prove that over-expression of miR-206 increased the percentage of cells at the GO-G1 transition and decreased the percentage of cells in the S phase and at the G2-M transition, as compared with the that in the miR-ctrl group (**Figure 2E** and **2F**). Additionally, we performed western blotting to demonstrate that the G1 phase-related proteins CDK2 and cyclin D1 were significantly down-regulated in miR-206over-expressing U87 and LN229 cells (**Figure 2G**). Taken together, these data suggested that miR-206 suppressed glioma cell growth *in vitro*.

Over-expression miR-206 suppresses GBM cell migration and invasion

Next, to identify the role of abnormal miR-206 expression in the regulation of glioma cell migration and invasion, in vitro analyses were performed on LV-miR-206- or LV-miR-ctrltransduced U87 and LN229 cells. First, the wound-healing scratch assay showed that the migratory ability of the cells over-expressing miR-206 was significantly lower than that of their corresponding controls (Figure 3A). Transwell assays then revealed that the invasive ability of U87 and LN229 cells was significantly diminished by miR-206 up-regulation (Figure **3B**). The invasive ability of the transduced cell lines was also determined in 3D spheroid assays. These results suggested that cells with miR-206 up-regulation had a weaker invasive ability than control cells did (Figure 3C). Furthermore, the expression of MMP9 and MMP2, two cancer invasion-associated proteins, was measured by western blotting. MMP9 and MMP2 expression clearly diminished after miR-206 over-expression. Because epithelial-tomesenchymal transition (EMT) is a critical process in cancer progression and affects cell migration and invasion, the expression levels of the mesenchymal markers (Slug, N-cadherin, and vimentin) were assessed by western blotting. All these markers were down-regulated in miR-206-over-expressing U87 and LN229 cells (Figure 3D). Thus, over-expression of miR-206 inhibited the migration and invasiveness of the human GBM cell lines.

MiR-206 directly targets FZD7 mRNA and suppresses the WNT pathway activity

Activation of the WNT pathway has been studied in various tumour types. It is widely accepted that β -catenin is an important downstream effector of WNT/ β -catenin signalling for the regulation of cell proliferation, migration, and invasion. Bioinformatics tools TargetScan, miR-



Figure 2. Over-expression of miR-206 attenuates GBM cell proliferation. A. MiR-206 expression was quantified by RT-qPCR in U87 and LN229 cells after transfection. B. Their proliferative ability was determined in the CCK-8 assay after cultivation for 96 h. C. The colony formation assay confirmed prolonged cell viability. D. Cell proliferation was also examined by the EdU incorporation assay. (Representative images are shown; the scale bar is 200 μ m.) E, F. The cell cycle distribution of U87 and LN229 cells that were transduced with an miR-206-expressing or negative control (miR-ctrl)-expressing lentivirus was analysed by flow cytometry. G. Western blotting of CDK2 and cyclin D1 in U87 and LN229 cells 48 h after transfection. Tubulin served as the loading control. (Data are presented as the means of triplicate experiments; #P < 0.0001, ***P < 0.001).



Figure 3. Over-expression of miR-206 suppresses GBM cell invasion and migration. A. Migration of GBM cells was assessed by a wound-healing scratch assay. Respective images of GBM cells transfected with either miR-ctrl or miR-206 (original magnification, 200×). B. Matrigel invasion assays uncovered the impact of miR-206 over-expression

on GBM cell invasion. C. GBM cell migration was monitored in a 3D spheroid migration assay (scale bar, 100 μ m). D. Invasion-associated proteins and EMT-associated proteins in U87 and LN229 cells after transfection with miR-ctrl or miR-206 were quantified by western blotting. Tubulin served as the loading control. (Data are presented as the means of triplicate experiments; ****P* < 0.001).

NAWaklk 2.0, DIANAmT, and PITA were employed to identify potential targets of miR-206 (Figure 4A). FZD7 was identified as one of the potential target genes of miR-206. In The Cancer Genome Atlas (TCGA) databases GSE-4290 and GSE16011, FZD7 expression was found to be significantly lower in all GBM subtypes than in NBT samples (Figure S1). Western blotting analysis showed that the expression levels of FZD7, β-catenin, and c-Myc were lower in miR-206-transfected U87 and LN229 cells (Figure 4B). Hence, we concluded that the WNT pathway was inhibited by miR-206. Next, we executed miRNA-related algorithms to identify the miR-206-binding sites in the 3'UTR of FZD7 mRNA (Figure 4C). We also performed immunofluorescence analysis of FZD7 and β-catenin in glioma cells. We observed that the decrease of β-catenin was accompanied by the decline of FZD7 in cells transfected with miR-206 (Figure 4D). To further investigate the correlation between miR-206 and FZD7, the levels of the FZD7 protein in glioma tissue samples and NBT samples were examined. As the results indicated, FZD7 was over-expressed in glioma tissue samples, and its expression level positively correlated with the WHO grade of the glioma (Figure 4E). We next assessed the correlation between FZD7 and miR-206 in the GBM samples. Spearman's correlation analysis suggested that FZD7 levels in GBM tissue samples inversely correlated with miR-206 levels (r = -0.42, Figure 4F and 4G). To confirm that FZD7 is a direct target of miR-206, luciferase reporter assays were performed. U87 and LN229 cells were co-transfected with vectors harbouring a wild-type or mutant FZD7 3'-UTR (Figure **4H**) and the miR-206 mimic. After transfection, ectopic miR-206 expression significantly suppressed luciferase activity resulting from the WT reporter but not from the mutant reporter. This finding suggested that miR-206 directly targeted the 3'-UTR of FZD7 mRNA and that point mutations in this sequence abrogated this interaction (Figure 4I). Human Protein Atlas database was used to investigate the expression level of FZD7 protein. In this database, six processed immunohistochemical staining of glioma patients were chosen (three of HGG, three of LGG). The the results indicated FZD7 was up-expressed in HGG samples compared with LGG samples (**Figure 4J**). The above findings indicated that miR-206 directly regulated FZD7 expression by binding to a site (nt positions 485-491) in the 3'-UTR of *FZD7* mRNA and subsequently suppressed the WNT pathway activity.

Down-regulation of FZD7 promotes GBM cell proliferation, migration, and invasion

Our experiments confirmed that FZD7 is a direct target gene of miR-206 in gliomas. We then focused on whether FZD7 can regulate the progression of GBM. To test this notion, we knocked down endogenous FZD7 expression in U87 and LN229 cells with a specific small interfering RNA (siRNA). Western blotting analysis confirmed that FZD7 protein expression was successfully suppressed by the FZD7 siRNA, and the activity of the WNT pathway was inhibited too (Figure 5A). CCK-8, EdU (Figure 5B and 5D), and colony formation assays (Figure 5C) were conducted to evaluate the effects of the FZD7 knockdown on cell viability and growth. We found that the proliferation of GBM cells was significantly inhibited by the knockdown of FZD7. Additionally, we carried out flow-cytometric analysis to examine cell cycle distribution after transfection. As compared with the results obtained from si-NC cells, we noted that the subpopulation of cells at the GO-G1 transition increased, whereas the numbers of cells in the S phase and at the G2-M transition decreased after the siRNA silencing of FZD7 (Figure 5G and 5H). To confirm that the depletion of FZD7 significantly inhibited glioma cell migration and invasion, a wound-healing scratch assay (Figure 5E), Transwell assay, and 3D spheroid assay (Figure 5F and 5I) were performed. Furthermore, the expression of MMP9 and MMP2 was measured by western blotting. MMP9 and MMP2 expression was clearly diminished in si-FZD7 cells. (Figure 5J) Their results showed that the silencing of FZD7 suppressed the migration and invasion abilities of human glioma cells compared with the control groups. This result is consistent with the observed effects of miR-206 over-expression.





Figure 4. MiR-206 directly targets *FZD7* mRNA and inhibits the WNT pathway. A. The Venn diagram computationally predicting that miR-206 targets *FZD7* mRNA according to five prediction algorithms: TargetScan, miRwalk, DIANAMT, miRanda, and PITA. B. After transfection with miR-ctrl or miR-206, the expression levels of FZD7, β -catenin, and c-Myc were determined by western blotting. Tubulin served as the loading control. C. The predicted miR-206-binding sequence in the *FZD7* 3'-UTR. D. Immunofluorescence staining of FZD7 (green) and β -catenin (red) in U87 and LN229 cells after transfection with miR-ctrl or miR-206. E, F. The expression levels of FZD7 in NBT and glioma samples were determined by western blotting. The fold changes were normalised to tubulin. NBT samples (n = 6) were collected from patients undergoing brain trauma surgery. Low-grade samples (n = 15) were derived from grade I and II gliomas, whereas high-grade samples (n = 18) were derived from grade III and IV gliomas. G. Pearson's correlation analysis of the relative expression levels of miR-206 and relative protein levels of FZD7. H. MiR-206-binding sites in the 3'-UTR of *FZD7* with wild-type (WT) or mutated (MUT) sequences highlighted. I. The dual-luciferase reporter assay was performed on U87 and LN229 cells, which were co-transfected with the indicated WT or mutant 3'-UTR construct and the miR-206 mimic. J. FZD7 protein expression level was elevated in primary glioma patient samples as indicated by the Human Protein Atlas database (http://www.proteinatlas.org/) (The data shown are representative of three independent experiments, **P* < 0.0001, ***P* < 0.01, **P* < 0.5).

Re-introduction of FZD7 attenuates the anticancer effects of miR-206 on glioblastoma cells in vitro

Having demonstrated that FZD7 is a direct target gene of miR-206 and that over-expression of miR-206 inhibited the proliferation, migration, and invasiveness of GBM cells, we then tested whether FZD7 is a functional target of miR-206. Plasmid pcDNA3-FZD7 was transfected into U87 and LN229 cells stably expressing either miR-206 or miR-ctrl. Western blotting analysis confirmed that FZD7 was significantly down-regulated in the miR-206-transfected group but up-regulated in the FZD7-transfected group, compared to the control groups. Of note, the level of β-catenin and c-Myc correlated with the expression level of FZD7, suggesting that FZD7 reintroduction rescued the blocked WNT/ β-catenin signalling pathway (Figure 6A). Experiments were then performed to assess proliferation and cell cycle progression. Both cell proliferation and cell cycle assay data indicated that the restoration of FZD7 expression partially antagonised the anti-cancer effects of miR-206 (Figure 6B-D and 6F). Next, a wound-healing assay, Transwell assay, (Figure 6E, 6G and 6H) and 3D spheroid assay (Figure 6I) were performed to examine migratory and invasive

abilities of glioma cells. The results of these analyses showed that forced expression of FZD7 reversed the effects of miR-206 on the migration and invasiveness of glioma cells. Meanwhile, western blotting suggested that the decrease in the expression levels of the G1 phase-related proteins, invasion-associated proteins, and mesenchymal markers in miR-206-transfected cells was reversed by forced FZD7 expression (**Figure 6J-L**). These findings strongly indicated that *FZD7* is a functional target of miR-206 in GBM cells.

MiR-206 over-expression attenuates tumour growth in vivo

Considering the significant effects of miR-206 on GBM cells *in vitro*, we extended our study to a nude-mouse model of an intracranial tumour to test whether the GBM growth *in vivo* could be retarded by over-expression of miR-206. Before implantation, U87 cells were co-transfected with a lentivirus expressing luciferase together with miR-206 or miR-ctrl. The growth of intracranial tumours was significantly impeded by miR-206 over-expression, as determined on days 14, 21, and 28 after implantation (**Figure 7A**). Kaplan-Meier survival curves also suggested that the survival of nude mice injected with



Figure 5. Down-regulation of FZD7 had an effect similar to that of the over-expression of miR-206 in glioma cells. A. Western blotting analysis of FZD7, β-catenin, and c-Myc expression in U87 and LN229 cells after a knockdown of FZD7. B. The CCK-8 assay after cultivation for 96 h evaluated the proliferative ability of U87 and LN229 cells. C. Long-term cell viability was evaluated using a colony formation assay. D. Proliferation of cells was examined by the EdU incorporation assay. Repre-

sentative images are shown (original magnification 200×). E. Migration of GBM cells was monitored using a wound-healing assay. (original magnification, 200×). F. Matrigel invasion assays uncovered the impact of the knockdown of FZD7 on GBM cell invasion. G, H. The cell cycle distribution of U87 and LN229 cells after a knockdown of FZD7 was analysed by flow cytometry. I. Migration of U87 and LN229 cells was monitored in a 3D spheroid migration assay. J. Invasion-associated proteins MMP9 and MMP2 in U87 and LN229 cells after a knockdown of FZD7 were quantified by western blotting. Tubulin served as the loading control. (Three independent experiments per group. Scale bar, 100 μ m. ****P* < 0.001).





Figure 6. FZD7 re-introduction reverses the tumour-suppressive effect of miR-206 on GBM cells. A. A rescue experiment was conducted by introducing pcDNA3.1-FZD7 or pcDNA3.1 (in the presence or absence of ectopic miR-206 or miR-ctrl expression) into U87 and LN229 cells. Western blotting analysis of FZD7, β -catenin, and c-Myc in the indicated cells. Tubulin served as the loading control. B. The viability of glioma cells transfected with pcDNA3.1-FZD7 and miR-206 separately or together was evaluated in the CCK-8 assay. C. The cell proliferative potential was evaluated by the EdU assay, 48 h after co-transfection. D. A colony formation assay was performed to evaluate the long-term cell viability of GBM cells after co-transfection. E. Migration of GBM cells was monitored using a wound-healing assay. Respective images of GBM cells after co-transfection (original magnification, 200×). F. The cell cycle distribution of U87 and LN229 cells after co-transfection was analysed by flow cytometry. G, H. Matrigel invasion assays were performed to evaluate the invasive ability of GBM cells after co-transfection. I. 3D spheroid assays were performed on the indicated cells. J, K. Western blotting analysis illustrates the regulation of Invasion-associated proteins and EMT-associated proteins in the indicated cells. L. Western blotting analysis uncovered the effects on cell cycle-regulatory proteins in the indicated cells. (All experiments were performed in triplicate; ****P* < 0.001, ***P* < 0.5).



Figure 7. MiR-206 attenuated tumour growth in a murine intracranial xenograft model. A. U87 cells pre-treated with a miR-ctrl-expressing or miR-206-expressing lentivirus (and a lentivirus expressing luciferase) were implanted into the brain of nude mice, and tumour formation was assessed by means of a non-invasive bioluminescence imaging system. Bioluminescent images were captured on days 0, 7, 14, 21, and 28 after the implantation. B. Overall survival was determined via Kaplan-Meier survival curves, and a log-rank test was conducted to assess the statistical significance of survival differences. C. After euthanasia, mouse brain tissues were collected, embedded into paraffin, and cut into thin sections for IHC analysis. The expression of FZD7, β -catenin, MMP2, and Ki-67 significantly diminished in the LV-miR-206 group.

miR-206-over-expressing cells was significantly longer (**Figure 7B**). Furthermore, IHC experiments implied that the tumours derived from miR-206-over-expressing U87 cells had lower FZD7 levels and significantly lower levels of β -catenin, Ki-67 and MMP2 (**Figure 7C**), in agreement with our *in vitro* results. In conclusion, these findings suggested that miR-206 exerted an obvious inhibitory effect on the tumorigenesis of GBM *in vivo* by repressing FZD7 expression.

Discussion

Mounting evidence indicates that miRNAs are closely related to the tumorigenesis of human cancers, including gliomas, and can serve as molecular biomarkers for tumour diagnosis and prognosis [9]. Some studies suggest that miR-206 dysregulation is associated with hepatocellular carcinoma, osteosarcoma, prostate cancer, breast cancer, and cervical cancer [11-14]. However, the expression pattern of miR- 206 in glioma is largely unknown. In this study, we investigated the biological roles of miR-206 and of its target gene, *FZD7*, in glioma cell proliferation, migration, and invasion. By searching the CGGA database, we found that miR-206 expression is significantly decreased in gliomas and negatively correlates with glioma grade. We then randomly selected 33 glioma tissue samples of different grades and six NBT samples to confirm the expression level of miR-206 in glioma tissue. RT-qPCR results implied that miR-206 expression was significantly lower in glioma tissue samples than in NBT samples. Furthermore, we found that miR-206 expression negatively correlates with glioma grade.

There is emerging evidence that the abnormal expression of miRNAs is associated with malignant biological behaviours, such as cell invasion, apoptosis, angiogenesis, and excessive cell proliferation [27-29]. To investigate these phenomena in glioma cells, we over-expressed miR-206 by means of a lentiviral vector. CCK-8, colony formation, and EdU incorporation assays were then conducted to assess the influence of miR-206 over-expression on the proliferation capacity of glioma cells. We also noted that miR-206 attenuates the migration and invasiveness of glioma cells. EMT has been identified as a vital regulatory process facilitating GBM cell invasion and migration [30]. The overexpression of miR-206 inhibits EMT signalling pathways in GBM cells [31]. Transwell invasion, wound healing, and in vitro 3D migration assays were carried out to investigate the migratory and invasive abilities of the transduced glioma cells. The results uncovered significantly decreased cell migration and invasion, both shortand long-term, after over-expression of miR-206. Furthermore, flow-cytometric assays indicated that miR-206 arrested cells at the G1-S transition and therefore suppressed proliferation. It is universally acknowledged that a protein kinase complex regulates the cell cycle. The cyclin E-CDK2 complex has been reported to perform a key function in the exit from GO-G1 transition and entry into the S phase [32, 33]. Our western blotting analysis showed that CDK2 and cyclin D1 were significantly downregulated upon over-expression of miR-206 or knockdown of FZD7. Therefore, we propose that the cell cycle can be arrested at the GO-G1 transition by miR-206 over-expression.

The WNT/ β -catenin pathway has been reported to often participate in the progression of

various types of cancers, including lung cancer, breast cancer, and prostate cancer. Furthermore, there is growing interest in potentially crucial participation of the WNT/β-catenin pathway in the pathogenesis of malignant glioma as well as in therapeutic targeting of this pathway [18]. FZD7 belongs to the FZD family of receptors and serves as a WNT protein receptor [34]. It is a crucial receptor in the WNT/β-catenin pathway. For instance, overexpression of microRNA-485-5p reduces migration and invasiveness of melanoma cells via down-regulation of FZD7 [27]. However, the relation between FZD7 and the clinical features of glioma remains unknown. In this study, we demonstrated that the FZD7 protein is over-expressed in glioma tissue samples and cell lines. Its expression was found to negatively correlate with miR-206 expression among clinical glioma specimens. Meanwhile, by luciferase reporter assays, we proved that oncogene FZD7 is a direct target of miR-206. Furthermore, the knockdown of FZD7 had an effect similar to that of miR-206 up-regulation in GBM cells. By contrast, subsequent re-introduction of FZD7 reversed the inhibition of glioma cell proliferation, migration, and invasion and changes in the cell cycle of glioma that were caused by up-regulated miR-206. In conclusion, our findings provide the first evidence that miR-206 significantly inhibits glioma cell progression by targeting FZD7 mRNA and thereby blocking the WNT/ β -catenin pathway.

In summary, our current data clearly provide novel evidence of a crucial link between miR-206 and the tumorigenesis of human glioma. These results suggest that miR-206 plays a key inhibitory role in the progression of GBM and functions as a tumour suppressor by down-regulating FZD7. The formation and progression of glioma are impaired by the miR-206/FZD7mediated blockage of the WNT/β-catenin pathway. Given that miRNA-based therapeutics are still in the initial stages of development, this novel finding not only improves our knowledge of the molecular mechanisms underlying glioma cell proliferation, migration, and invasion but also points to a promising therapeutic strategy against glioma.

Acknowledgements

This work was supported by grants from the National Key Research and Development Plan (2016YFC0902500), the National Natural Sci-

ence Foundation of China (81672501 and 81-872058), Jiangsu Province's Natural Science Foundation (20170108), the Program for Advanced Talents within Six Industries of Jiangsu Province (2015-WSN-036 and 2016-WSW-013), Jiangsu Province's Key Discipline of Medicine (ZDXKA2016001), and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Disclosure of conflict of interest

None.

Address correspondence to: Chunsheng Zhao and Yingyi Wang, Department of Neurosurgery, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, Jiangsu Province, China. E-mail: nwzhaochunsheng@126.com (CSZ); Lindsay_wang-3431@126.com (YYW)

References

- [1] Ostrom QT, Bauchet L, Davis FG, Deltour I, Fisher JL, Langer CE, Pekmezci M, Schwartzbaum JA, Turner MC, Walsh KM, Wrensch MR and Barnholtz-Sloan JS. The epidemiology of glioma in adults: a "state of the science" review. Neuro Oncol 2014; 16: 896-913.
- [2] Rasmussen BK, Hansen S, Laursen RJ, Kosteljanetz M, Schultz H, Norgard BM, Guldberg R and Gradel KO. Epidemiology of glioma: clinical characteristics, symptoms, and predictors of glioma patients grade I-IV in the the Danish neuro-oncology registry. J Neurooncol 2017; 135: 571-579.
- [3] Ohgaki H and Kleihues P. Epidemiology and etiology of gliomas. Acta Neuropathol 2005; 109: 93-108.
- [4] Van Meir EG, Hadjipanayis CG, Norden AD, Shu HK, Wen PY and Olson JJ. Exciting new advances in neuro-oncology: the avenue to a cure for malignant glioma. CA Cancer J Clin 2010; 60: 166-193.
- [5] Luthra PM and Lal N. Prospective of curcumin, a pleiotropic signalling molecule from Curcuma longa in the treatment of Glioblastoma. Eur J Med Chem 2016; 109: 23-35.
- [6] Esquela-Kerscher A and Slack FJ. Oncomirs microRNAs with a role in cancer. Nat Rev Cancer 2006; 6: 259-269.
- [7] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-297.
- [8] Lewis BP, Burge CB and Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 2005; 120: 15-20.

- [9] Areeb Z, Stylli SS, Koldej R, Ritchie DS, Siegal T, Morokoff AP, Kaye AH and Luwor RB. MicroRNA as potential biomarkers in Glioblastoma. J Neurooncol 2015; 125: 237-248.
- [10] Zhi T, Jiang K, Xu X, Yu T, Wu W, Nie E, Zhou X, Jin X, Zhang J, Wang Y and Liu N. MicroRNA-520d-5p inhibits human glioma cell proliferation and induces cell cycle arrest by directly targeting PTTG1. Am J Transl Res 2017; 9: 4872-4887.
- [11] Pang C, Huang G, Luo K, Dong Y, He F, Du G, Xiao M and Cai W. miR-206 inhibits the growth of hepatocellular carcinoma cells via targeting CDK9. Cancer Med 2017; 6: 2398-2409.
- [12] Ren D, Zheng H, Fei S and Zhao JL. MALAT1 induces osteosarcoma progression by targeting miR-206/CDK9 axis. J Cell Physiol 2018; 234: 950-957.
- [13] Wang Y, Xu H, Si L, Li Q, Zhu X, Yu T and Gang X. MiR-206 inhibits proliferation and migration of prostate cancer cells by targeting CXCL11. Prostate 2018; 78: 479-490.
- [14] Wang R, Zhang T, Yang Z, Jiang C and Seng J. Long non-coding RNA FTH1P3 activates paclitaxel resistance in breast cancer through miR-206/ABCB1. J Cell Mol Med 2018; 22: 4068-4075.
- [15] Wang Y and Tian Y. miR-206 inhibits cell proliferation, migration, and invasion by targeting BAG3 in human cervical cancer. Oncol Res 2018; 26: 923-931.
- [16] Ahmad I, Morton JP, Singh LB, Radulescu SM, Ridgway RA, Patel S, Woodgett J, Winton DJ, Taketo MM, Wu XR, Leung HY and Sansom OJ. beta-catenin activation synergizes with PTEN loss to cause bladder cancer formation. Oncogene 2011; 30: 178-189.
- [17] Chen Q, Cai J, Wang Q, Wang Y, Liu M, Yang J, Zhou J, Kang C, Li M and Jiang C. Long noncoding RNA NEAT1, regulated by the EGFR pathway, contributes to glioblastoma progression through the WNT/beta-catenin pathway by scaffolding EZH2. Clin Cancer Res 2018; 24: 684-695.
- [18] McCord M, Mukouyama YS, Gilbert MR and Jackson S. Targeting WNT signaling for multifaceted glioblastoma therapy. Front Cell Neurosci 2017; 11: 318.
- [19] Valenta T, Hausmann G and Basler K. The many faces and functions of beta-catenin. EMBO J 2012; 31: 2714-2736.
- [20] Sagara N, Toda G, Hirai M, Terada M and Katoh M. Molecular cloning, differential expression, and chromosomal localization of human frizzled-1, frizzled-2, and frizzled-7. Biochem Biophys Res Commun 1998; 252: 117-122.
- [21] Ueno K, Hirata H, Hinoda Y and Dahiya R. Frizzled homolog proteins, microRNAs and Wnt

signaling in cancer. Int J Cancer 2013; 132: 1731-1740.

- [22] Merle P, de la Monte S, Kim M, Herrmann M, Tanaka S, Von Dem Bussche A, Kew MC, Trepo C and Wands JR. Functional consequences of frizzled-7 receptor overexpression in human hepatocellular carcinoma. Gastroenterology 2004; 127: 1110-1122.
- [23] Kim M, Lee HC, Tsedensodnom O, Hartley R, Lim YS, Yu E, Merle P and Wands JR. Functional interaction between Wnt3 and Frizzled-7 leads to activation of the Wnt/beta-catenin signaling pathway in hepatocellular carcinoma cells. J Hepatol 2008; 48: 780-791.
- [24] Ueno K, Hiura M, Suehiro Y, Hazama S, Hirata H, Oka M, Imai K, Dahiya R and Hinoda Y. Frizzled-7 as a potential therapeutic target in colorectal cancer. Neoplasia 2008; 10: 697-705.
- [25] Li G, Su Q, Liu H, Wang D, Zhang W, Lu Z, Chen Y, Huang X, Li W, Zhang C, He Y, Fu L and Bi J. Frizzled7 promotes epithelial-to-mesenchymal transition and stemness via activating canonical wnt/beta-catenin pathway in gastric cancer. Int J Biol Sci 2018; 14: 280-293.
- [26] Yang L, Wu X, Wang Y, Zhang K, Wu J, Yuan YC, Deng X, Chen L, Kim CC, Lau S, Somlo G and Yen Y. FZD7 has a critical role in cell proliferation in triple negative breast cancer. Oncogene 2011; 30: 4437-4446.
- [27] Wu J, Li J, Ren J and Zhang D. MicroRNA-485-5p represses melanoma cell invasion and proliferation by suppressing Frizzled7. Biomed Pharmacother 2017; 90: 303-310.
- [28] Zeng A, Yin J, Li Y, Li R, Wang Z, Zhou X, Jin X, Shen F, Yan W and You Y. miR-129-5p targets Wnt5a to block PKC/ERK/NF-kappaB and JNK pathways in glioblastoma. Cell Death Dis 2018; 9: 394.

- [29] Li Y, Wang Y, Yu L, Sun C, Cheng D, Yu S, Wang Q, Yan Y, Kang C, Jin S, An T, Shi C, Xu J, Wei C, Liu J, Sun J, Wen Y, Zhao S and Kong Y. miR-146b-5p inhibits glioma migration and invasion by targeting MMP16. Cancer Lett 2013; 339: 260-269.
- [30] Zeng AL, Yan W, Liu YW, Wang Z, Hu Q, Nie E, Zhou X, Li R, Wang XF, Jiang T and You YP. Tumour exosomes from cells harbouring PT-PRZ1-MET fusion contribute to a malignant phenotype and temozolomide chemoresistance in glioblastoma. Oncogene 2017; 36: 5369-5381.
- [31] Jiao D, Chen J, Li Y, Tang X, Wang J, Xu W, Song J, Li Y, Tao H and Chen Q. miR-1-3p and miR-206 sensitizes HGF-induced gefitinib-resistant human lung cancer cells through inhibition of c-Met signalling and EMT. J Cell Mol Med 2018; 22: 3526-3536.
- [32] Cheney IW, Neuteboom ST, Vaillancourt MT, Ramachandra M and Bookstein R. Adenovirusmediated gene transfer of MMAC1/PTEN to glioblastoma cells inhibits S phase entry by the recruitment of p27Kip1 into cyclin E/CDK2 complexes. Cancer Res 1999; 59: 2318-2323.
- [33] Kaldis P and Aleem E. Cell cycle sibling rivalry: Cdc2 vs. Cdk2. Cell Cycle 2005; 4: 1491-1494.
- [34] Flanagan DJ, Phesse TJ, Barker N, Schwab RH, Amin N, Malaterre J, Stange DE, Nowell CJ, Currie SA, Saw JT, Beuchert E, Ramsay RG, Sansom OJ, Ernst M, Clevers H and Vincan E. Frizzled7 functions as a Wnt receptor in intestinal epithelial Lgr5(+) stem cells. Stem Cell Reports 2015; 4: 759-767.



Figure S1. A-C. The transcriptional levels of *FZD7* were analysed in non-brain tissues (NBT), low-grade gliomas (LGG) and high-grade gliomas (HGG) from the TCGA, GSE4290, and GSE16011 databases.