

Research Article

Dysregulation of Fra1 expression by Wnt/ β -catenin signalling promotes glioma aggressiveness through epithelial–mesenchymal transition

Li Zhang¹, Huaijun Liu², Xiaodan Mu², Jianling Cui¹ and Zhigang Peng¹¹Department of CT/MRI, The Third Hospital of Hebei Medical University, Shijiazhuang 050051, Hebei Province, China; ²Department of Medical Imaging, The Second Hospital of Hebei Medical University, Shijiazhuang 050000, Hebei Province, China

Correspondence: Huaijun Liu (lhj6254@foxmail.com)



Aberrant expression of Fos-related antigen-1 (Fra1) is commonly elevated in various malignant cancers and is strongly implicated in invasion and metastasis. However, the molecular mechanisms underlying its dysregulation in human glioma remain poorly understood. In the present study, we demonstrate that up-regulation of Fra1 plays a crucial role in the glioma aggressiveness and epithelial–mesenchymal transition (EMT) activated by Wnt/ β -catenin signal pathway. In glioma cells, activation of Wnt/ β -catenin signalling by Wnt3a administration obviously induced EMT and directly activated the transcription of Fra1. Phenotype experiments revealed that up-regulation of Fra1 induced by Wnt/ β -catenin signalling drove the EMT of glioma cells. Furthermore, it was found that the cisplatin resistance acquired by Wnt/ β -catenin signalling activation depended on increased expression of Fra1. Analysis of clinical specimens verified a positive correlation between Fra1 and β -catenin as well as a poor prognosis in glioma patients with double-high expressions of them. These findings indicate that an aberrant Wnt/ β -catenin signalling leads to the EMT and drug resistance of glioma via Fra1 induction, which suggests novel therapeutic strategies for the malignant disease.

Introduction

Among various human cancers, malignant glioma (MG) is one of the most aggressive tumours and occurs frequently in the central nervous system [1]. MG often results in a high mortality rate and poor prognosis because of its highly invasive and infiltrative character [2]. In most cases, lethality in glioma patients is caused by metastasis involving migration and invasion of cancer cells, which results in resistance to the existing drugs [3]. Although many studies have demonstrated the knowledge of invasive and infiltrative properties in glioma, the mechanisms for metastasis are still not well determined but necessary for prognosis and treatment.

Numerous factors and signal pathways associated with the invasion and migration of glioma cancer have been found in previous studies [4]. Among them, epithelial–mesenchymal transition (EMT), which reduces epithelial markers (E-cadherin) and increases mesenchymal markers (N-cadherin and Vimentin), leads to an increased invasive or metastatic phenotype in glioma cells [5]. Besides, it has been reported that the EMT-related transcription factors (EMT-TFs) including Slug, Snail, ZEB1 and Twist are indispensable for EMT process [6–9]. As another important factor, Fos-related antigen-1 (Fra1) always indicates metastasis and poor prognosis in a variety of human cancers. Different from other members of the Fos family, Fra1 is closely related with the motile and invasive phenotypes of cancer cells [10]. Mesenchymal characteristics and morphological changes could be induced by overexpression of Fra1 [11]. More recently, Fra1

Received: 29 December 2016
Revised: 12 February 2017
Accepted: 22 February 2017Accepted Manuscript Online:
23 February 2017
Version of Record published:
27 March 2017

has been demonstrated as a gatekeeper of the EMT programme during cancer progression [12,13]. However, the mechanism for Fra1 regulation in glioma remains unclear.

The Wnt/ β -catenin pathway plays a critical role in many biological processes such as cell survival, proliferation, migration and polarity [14]. Aberrant activation of Wnt/ β -catenin signalling has been observed in a variety of human cancers including glioma. At the molecular level, Wnt ligands interact with Fzd/LRP that induce the translocation of β -catenin from cytoplasm to nucleus. The nucleus-accumulated β -catenin functions as a transcriptional co-activator with Tcf/Lef and then regulates the expressions of target genes such as *c-Myc*, *cyclin D1* and matrix metalloproteinases (MMPs) [15,16]. β -catenin, a key signal transducer of Wnt pathway, is overexpressed in glioma and knockdown of it reduces the invasiveness of glioma cells [17]. Although previous findings confirmed Wnt/ β -catenin signalling as a metastasis driver in glioma, more target genes need to be identified to better understand the pathway [18].

In the present study, Fra1 is identified as a target of Wnt/ β -catenin signalling and then mediates EMT as well as cisplatin resistance in glioma cells. Further clinical analysis reveals that Fra1 is positively correlated with β -catenin and glioma progression, which confirms a critical role of Wnt/ β -catenin/Fra1 signalling axis in glioma aggressiveness.

Materials and methods

Cell culture and treatment

Human glioma cell lines U-251 and U-87 were purchased from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO₂. For lithium chloride (LiCl) or CHIR99021 treatment, cells were cultured to approximately 40% confluence and then treated with 20 mM LiCl or 3 μ M CHIR99021. Transfections of plasmids and siRNA were performed using Lipofectamine 2000 transfection reagent (Life Technologies) according to the manufacturer's instructions. Stable transfections of Wnt3a and shFra1 were maintained by G418.

Plasmids, siRNA and antibodies

The constructs encoding Flag-tagged Fra1 and Wnt3a were cloned into pcDNA3.0 vector. The siRNA (sc-35405) and shRNA (sc-35405-SH) targeting Fra1 were obtained from Santa Cruz Biotechnology. Antibodies against E-cadherin (sc-59780), Fibronectin (sc-69681), Vimentin (sc-73259), ZO-1 (sc-8146), Fra1 (sc-48424), Zeb2 (sc-271984), β -catenin (sc-31001) and GAPDH (sc-51907) were from Santa Cruz. Antibodies against Twist1 (ab50581), Zeb1 (ab124512), Snail (ab53519), Slug (ab27568) were from Abcam.

Quantitative real-time PCR analysis

Isolation of total RNA from cells was performed using RNAiso Plus (TaKaRa). The purity and concentration of RNA samples were determined by NanoDrop Spectrophotometer (NanoDrop). Reverse transcription was carried out with the PrimeScript RT reagent kit (TaKaRa). Real-time quantitative PCR was achieved using SYBR Select Master Mix (Roche) according to the manufacturer's instructions. Target gene expression was normalized to actin levels in respective samples as an internal control and the results are representative of at least three independent experiments.

Western blot

Cells were harvested and lysed using Ripa lysis buffer (50 mM Tris/HCl, pH 7.4, 100 mM 2-mercaptoethanol, 2% w/v SDS, 10% glycerol). The protein concentration was determined with the Bradford method (Bio-Rad). The proteins were separated by 10% SDS/PAGE and transferred to nitrocellulose membranes (Whatman). The membranes were incubated with dilutions of primary antibodies followed by IRDye 800CW or IRDye 680-conjugated secondary antibodies and then detected by the Odyssey Infrared Imaging System (Li-COR).

Luciferase reporter assay

The promoter fragments of human Fra1 were amplified by PCR and cloned into the pGL3 vector. The reporter constructs containing different lengths of Fra1 promoter or mutated Tcf/Lef-binding sites were generated by subsequent PCR-based cloning. A pair of luciferase reporter constructs, TOPflash and FOPflash, was used to evaluate Tcf/Lef transcriptional activity. TOPflash contains three copies of TCF-4-binding sites and FOPflash contains mutated TCF-4-binding sites. The pRL-SV40 vector was cotransfected with the reporter constructs in each experiment as an internal control for transfection efficiency. After transfection for 24 h, cells were treated with 200 ng/ml Wnt3a for 12 h and luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). All experiments were performed in triplicate.

ChIP

Chromatin was cross-linked with 1% formaldehyde and sonicated to obtain a DNA fragment of 200–500 bp. After centrifugation, the supernatants were subjected to immunoprecipitation overnight at 4°C with antibodies against β -catenin or normal IgG. The DNA–protein complexes were isolated using Protein A/G PLUS-Agarose (Santa Cruz). The cross-linking was reversed and released DNA fragments were purified and quantified by QPCR using the following primer pairs for Fra1 promoter: (Forward) ACCGTAATGAAGATGGCG; (Reverse) TTAAATGTTTGCGATAAGG. Cmyc promoter [19]: (Forward) GGTCCACAAGCTCTCCACTT; (Reverse) CGGTTTGCAACAGTCTCG. Control: (Forward) TGAGATTAGAATTGGGACAG; (Reverse) TAGAGACAGGGTCTCAC-TAG. Three independent experiments were performed.

Cell viability assay

U-251 cells in logarithmic growth were plated and treated with the indicated reagents or transfections. After 48 h of culture, CCK-8 (cell counting kit-8) (Dojindo Molecular Technologies, MD, U.S.A.) was added and the OD₄₅₀ was measured using an automatic plate reader.

Wound healing assay

Cell culture and transfection were performed as described above. At 24 h after transfection, the monolayer was gently and slowly aspirated with a micropipette-like tip to create a 1 mm cell-free zone that was washed with serum-free medium or PBS to remove any remaining cells. The cell-free area was marked, serum-free medium was added, and the cells were returned to the incubator for 24–48 h. Images were acquired at different time points during the incubation. The wound was evaluated using ImageJ.

Cell invasion assay

Cell invasion assay was performed in 24-well transwell plates (Costar) with 8 μ m pore inserts coated with Matrigel (BD Biosciences). U-251 and U-87 cells (1×10^5) were plated to a culture insert in serum-free medium, whereas complete medium with or without 200 ng/ml Wnt3a was applied to the lower compartment. After incubation for 48 h, the undersurface adherent cells that had invaded through the Matrigel were fixed in methanol and stained with 0.5% Crystal Violet. The air dried filter membrane was viewed under a microscope and four random fields were selected for cell counting.

Flow cytometric detection of apoptosis

U-251 and U-87 cells were collected for detection of apoptosis using the Annexin-V-FLUOS Kit (Roche, U.S.A.) according to the manufacturer's instructions. All the samples were assayed in triplicate.

Xenograft tumour assay

In vivo chemosensitivity experiment was performed by the subcutaneous transplantation of cells into BALB/c nude mice. The U-251 cells stably transfected with Wnt3a expression plasmids and shFra1 were suspended at a density of 10^7 cells/ml and 100 μ l was injected into the flank of each nude mouse ($n=6$). Ten days post-injection, cisplatin (5 mg/kg) was intraperitoneally administered every 3 days for 18 days. Seven days after subcutaneous transplantation, we measured the sizes of the growing tumours every 3 days for 21 days using the formula ($W^2 \times L$). The mice were then killed and tumour weights were measured. All animal procedures were performed in compliance with the Guidelines for the Care and Use of Experimental Animals established by the Committee for Animal Experimentation of Hebei Medical University.

Immunohistochemistry

In total, 87 glioma tissue specimens were collected for the present study from 2005 to 2012 from The Second Hospital of Hebei Medical University and The Third Hospital of Hebei Medical University (Hebei, China). All specimens were fixed in 10% neutral formalin, embedded in paraffin and cut into 4- μ m sections for immunohistochemical staining. The EnVision™ two-step method was used (DAKO, Hamburg, Germany), as well as the following antibodies: antibodies against Fra1 and β -catenin, were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). Prior to staining, sections were pretreated with microwaves for 18 min in a 0.01 M citrate buffer (pH 6.0) for antigen retrieval. 3,3'-Diaminobenzidine was used as a chromogen and 0.01 M PBS (pH 7.4) was substituted for primary antibodies as a negative control. To estimate the score for each slide, at least eight individual fields at 200 \times were chosen and 100 cancer cells were counted in each field. The immunostaining intensity was divided into four grades: 0, no expression;

1, mildly positive; 2, moderately positive and 3, markedly positive. The proportion of positive-staining cells was divided into five grades: 0, <10%; 1, 11–25%; 2, 26–50%; 3, 51–75% and 4, >75%. The staining results were assessed and confirmed by two independent investigators blinded to the clinical data. The percentage of positivity of the tumour cells and the staining intensities were then multiplied in order to generate the IHC score and graded as low expression (score 0–6) and high expression (score 7–12). Cases with a discrepancy in scores were discussed to obtain a consensus.

Statistical analysis

The software SPSS 13.0 was used in statistical analyses. Group distributions were performed with the Student's *t* test or one-way ANOVA. Pearson's correlation test was used in analysing the correlation. A value of $P < 0.05$ was considered statistically significant.

Results

Activation of Wnt/ β -catenin signalling induces EMT in glioma cells

It is noteworthy to determine the potential mechanisms of Wnt/ β -catenin signalling in induction of glioma EMT. In U-251 and U-87 cells, decreased E-cadherin and ZO-1 accompanied by induction of Vimentin and Fibronectin at the mRNA level were observed in a Wnt3a dose-dependent manner (Figure 1A,B). Consistently, the protein levels of epithelial markers (E-cadherin and ZO-1) were repressed whereas mesenchymal markers (Vimentin and Fibronectin) increased after exogenous Wnt3a treatment (Figure 1C,D). Immunofluorescence showed that activation of Wnt/ β -catenin signalling by Wnt3a promoted the expression of Vimentin, whereas blocking Wnt/ β -catenin pathway by silencing β -catenin suppressed the mesenchymal marker in glioma cells (Figure 1E). As expected, knockdown of β -catenin attenuated Wnt3a induced up-regulation of Vimentin (Figure 1F,G). These results suggest a promotive effect of Wnt/ β -catenin signalling on glioma EMT. Furthermore, several EMT-TFs were screened by QPCR and Western blot after activation of Wnt/ β -catenin signalling. Although all the factors were elevated to different extents after Wnt3a treatment, both the mRNA and protein levels of Fra1 showed most significant up-regulation (Figure 1H–J). Therefore, Wnt/ β -catenin signalling enhances the EMT of glioma cells, which may be accomplished by inducing EMT-TFs.

Wnt/ β -catenin signalling activates the transcription of Fra1 in glioma cells

To further study the relationship between Wnt/ β -catenin signalling and Fra1, glioma cells were exposed to specific inhibitors (LiCl and CHIR99021) for GSK-3 β . As a result, activating Wnt/ β -catenin signalling by targeting GSK-3 β increased the mRNA and protein levels of Fra1 (Figure 2A,B). Consistently, luciferase reporter assay showed that Wnt3a exposure increased the transcriptional activity of Fra1 promoter (–2500/+1) in a dose-dependent manner (Figure 2C), and the accumulation of Fra1 was further confirmed at the protein level (Figure 2D). Promoter analysis of human Fra1 gene predicted three potential Tcf/Lef-binding sites at the proximal region of Fra1 promoter, and then serial deletion constructs of the Fra1 gene promoter were analysed to identify the transcriptional regulatory elements responsive to Wnt/ β -catenin signalling (Figure 2E). It was shown that the Fra1 promoter without the region between –1500 and –500 lost the ability to respond to Wnt3a treatment (Figure 2F). The DNA fragment (–1500/–500) contained two putative Tcf/Lef-binding sites. Mutation in the latter site (Site3: –660/–647) obviously decreased the reporter activity induced by Wnt3a (Figure 2G). ChIP experiment further demonstrated that β -catenin/Tcf/Lef transcriptional complex directly bound to the Fra1 promoter approximately –700 bp upstream of the transcription start site and the binding was strengthened after Wnt3a stimulation (Figure 2H,I). These data suggest that Wnt/ β -catenin signalling up-regulates Fra1 expression via transactivation.

Fra1 promotes Wnt3a-induced migration and invasion of glioma cancer cells

As expected, overexpression of Fra1 decreased the expressions of epithelial markers and increased the expressions of mesenchymal markers, whereas Fra1 knockdown had the opposite effect (Figure 3A). Considering increased invasion and migration as a classic feature of EMT, wound healing and cell invasion assays were performed to investigate the role of Fra1 in the EMT induced by Wnt/ β -catenin signalling. Knockdown of either Fra1 or β -catenin weakened the migratory and invasive abilities of U-251 cells (Figure 3B,C). In U-87 cells, knockdown of β -catenin impaired the promotive effect of Wnt3a on the aggressive phenotypes (Figure 3D,E). Moreover, the acquisition of migratory and

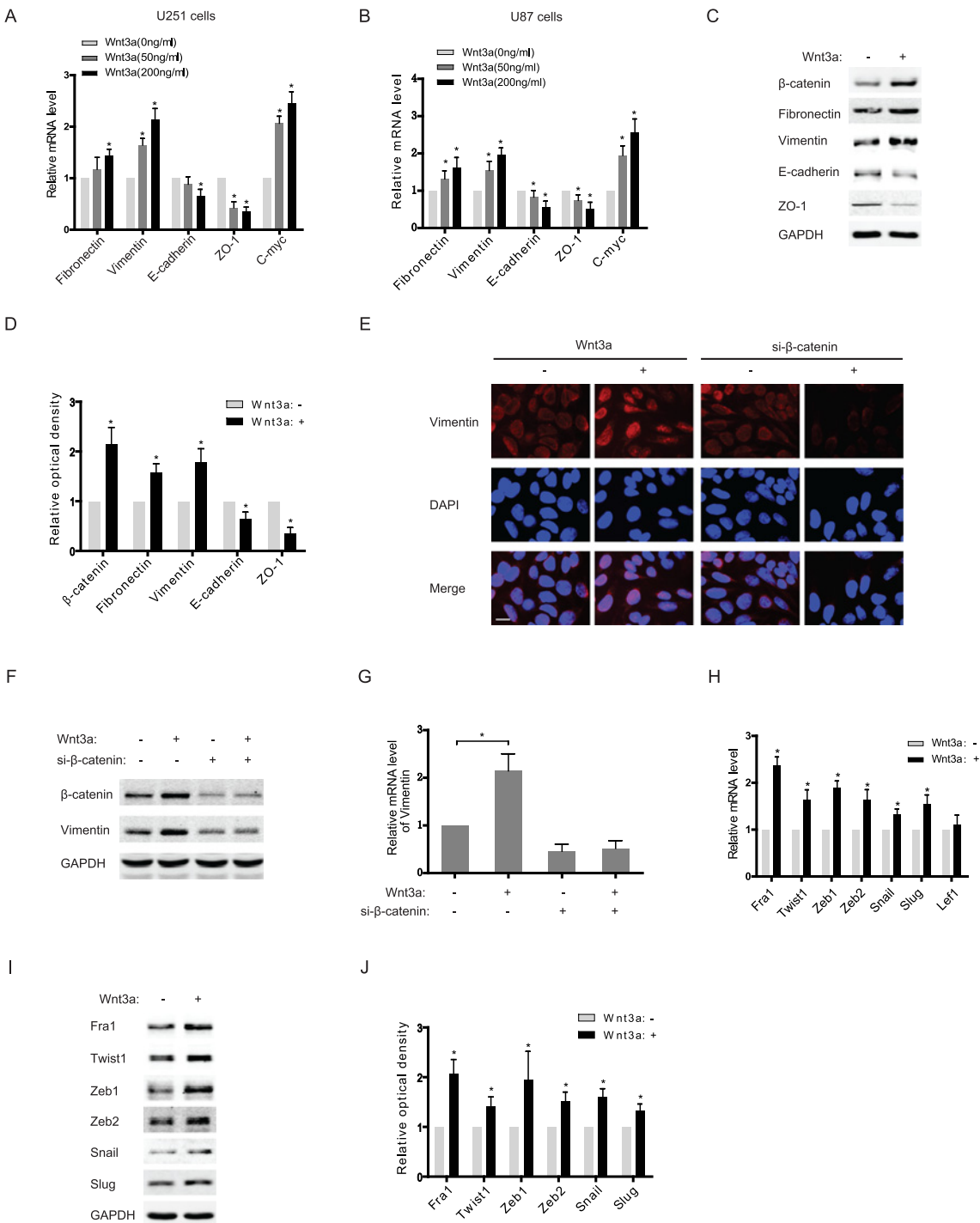


Figure 1. Activation of Wnt/β-catenin signalling induces EMT in glioma cells

(A, B) Relative mRNA levels of EMT markers in the U-251 (A) and U-87 (B) cells treated with Wnt3a at different concentrations (50 and 200 ng/ml) for 48 h; * $P < 0.05$. (C) Western blots of EMT markers with specific antibodies in the U-251 cells exposed to Wnt3a (200 ng/ml) for 48 h. (D) Quantificative analysis of (C) by Image J; * $P < 0.05$. (E) Immunofluorescent staining for Vimentin in the U-251 cells treated with Wnt3a (200 ng/ml) or transfected with si-β-catenin for 48 h (nuclei stained with DAPI) (Scale bar 20 μm). (F, G) The protein (F) and mRNA (G) levels of Vimentin in the U-251 cells with si-β-catenin transfection and Wnt3a (200 ng/ml) treatment; * $P < 0.05$. (H) Relative mRNA levels of EMT-TFs in the U-251 cells treated with Wnt3a (200 ng/ml) for 48 h. Lef1 served as a negative control; * $P < 0.05$. (I) Western blots of EMT-TFs with specific antibodies in the U-251 cells exposed to Wnt3a (200 ng/ml) for 48 h. (J) Quantificative analysis of (I) by Image J; * $P < 0.05$.

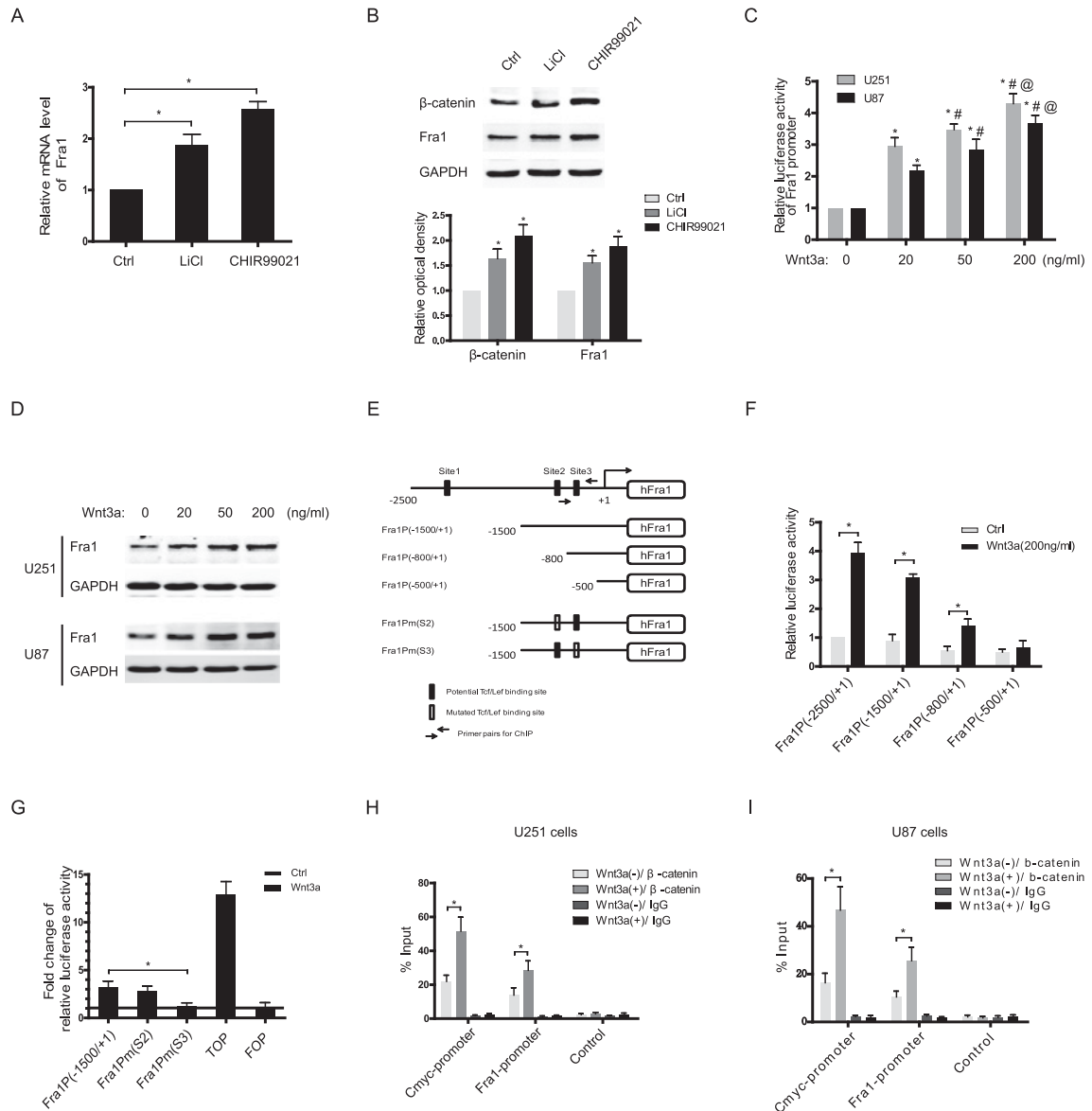


Figure 2. Wnt/ β -catenin signalling activates the transcription of Fra1 in glioma cells

(A) Relative mRNA levels of Fra1 in the U-251 cells treated with the GSK-3 β inhibitors, LiCl (20 mM) or CHIR (3 μ M) for 48 h; $^*P < 0.05$. (B) Protein levels of Fra1 and β -catenin in the U-251 cells treated with the GSK-3 β inhibitors, LiCl (20 mM) or CHIR (3 μ M) for 48 h; $^*P < 0.05$. (C) The transcriptional activity of Fra1 promoter (-2500/+1) in response to different concentrations (20, 50 and 200 ng/ml) of Wnt3a for 48 h was measured by luciferase assay in U-251 and U-87 cells (internal control, pRL-SV40). * Compared with 0 ng/ml; $^\#$ Compared with 20 ng/ml; $^\circ$ Compared with 50 ng/ml; $P < 0.05$. (D) The protein level of Fra1 in response to different concentrations (20, 50 and 200 ng/ml) of Wnt3a was detected by Western blot. (E) Schematic representation of Fra1 promoter with three potential Tcf/Lef-binding sites and the primer pair used in ChIP-PCR assays. The truncated and mutated derivatives are also shown. (F) Relative luciferase activities were measured with a series of truncated constructs of Fra1P in the U-251 cells exposed to Wnt3a (200 ng/ml) for 48 h (internal control, pRL-SV40, $^*P < 0.05$). (G) Relative luciferase activity in the U-251 cells transfected with wild-type Fra1P (-1500/+1) or Tcf/Lef-binding site mutated Fra1P (-1500/+1) in response to 48 h Wnt3a (200 ng/ml) treatment (internal control, pRL-SV40, $^*P < 0.05$). TOPflash containing wild-type TCF-4-binding sites were used as a positive control, whereas FOPflash with mutant-binding sites served as negative control. The results were normalized to the control group (without Wnt3a). (H, I) The U-251 and U-87 cells treated with Wnt3a (200 ng/ml) for 48 h were analysed by ChIP assays using β -catenin antibody and IgG. QPCR was performed with the immunoprecipitated DNAs or soluble chromatin using the specific primer pairs for Fra1 promoter. Cmyc or control served as a positive or a negative control. Error bars represent S.D. of three individual experiments.

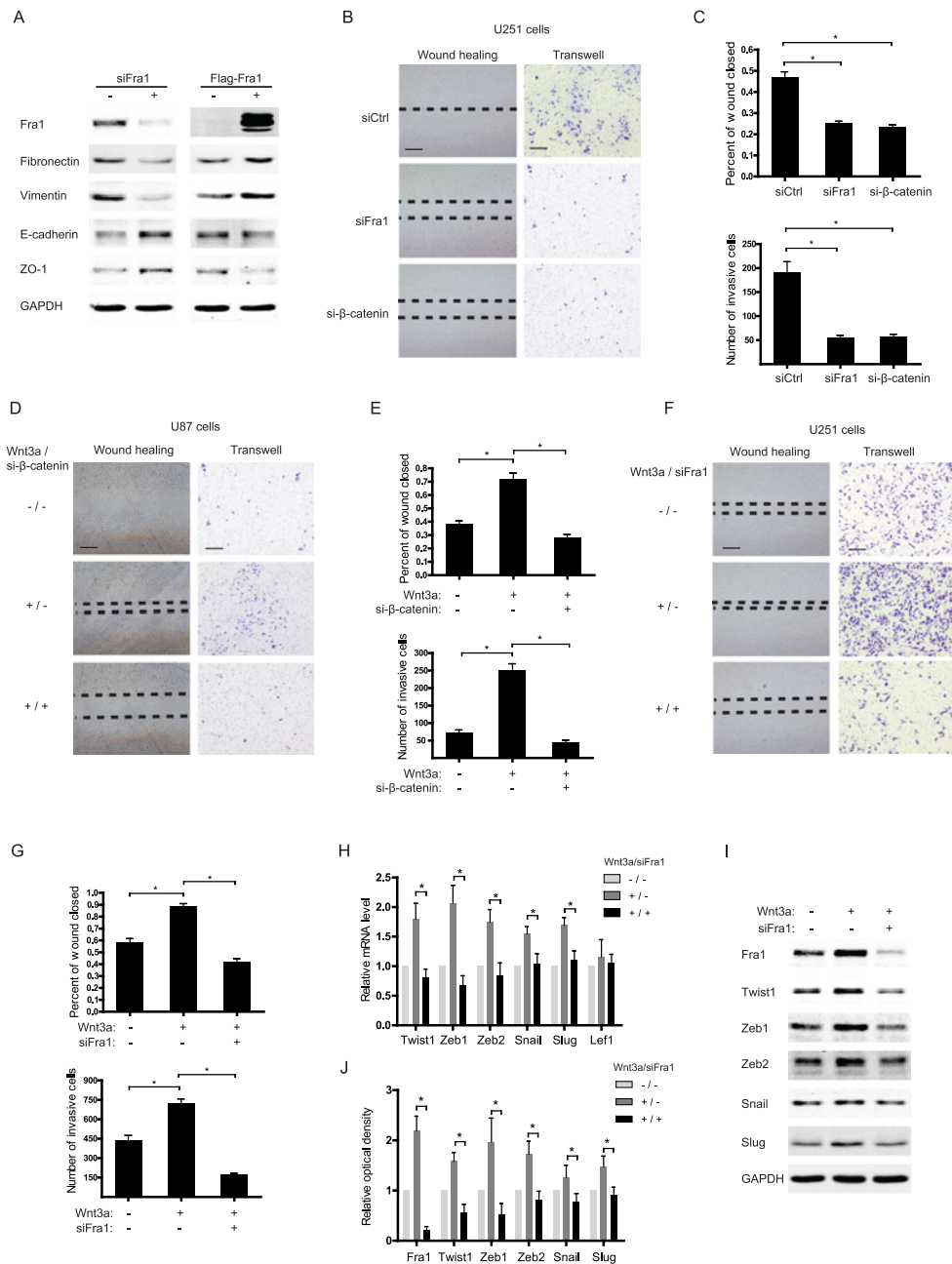


Figure 3. Fra1 promotes Wnt3a-induced migration and invasion of glioma cancer cells

(A) Western blots of EMT markers with specific antibodies in the U-251 cells transfected with Fra1 siRNA or expression vector. (B) Left panels: representative images from wound healing assays with the U-251 cells transfected with indicated siRNA. Right panels: representative images of the U-251 cells penetrating the Matrigel in invasion assays after transfection with indicated siRNA (Scale bar 200 μ m). (C) Up panel: percentage wound closure 48 h after scratch in (B). Down panel: numbers of invasive cells in (B); $*P < 0.05$. (D) Left panels: representative images from wound healing assays with the U-87 cells transfected with β -catenin siRNA and exposed to Wnt3a (200 ng/ml) for 48 h. Right panels: representative images of the U-87 cells penetrating the Matrigel in invasion assays after transfection with β -catenin siRNA and exposure to Wnt3a (200 ng/ml) for 48 h (Scale bar 200 μ m). (E) Up panel: percentage wound closure in (D). Down panel: numbers of invasive cells in (D); $*P < 0.05$. (F) Left panels: representative images from wound healing assays with the U-251 cells transfected with Fra1 siRNA and exposed to Wnt3a (200 ng/ml) for 48 h. Right panels: representative images of the U-251 cells penetrating the Matrigel in invasion assays after transfection with Fra1 siRNA and exposure to Wnt3a (200 ng/ml) for 48 h (Scale bar 200 μ m). (G) Up panel: percentage wound closure in (F). Down panel: numbers of invasive cells in (F); $*P < 0.05$. (H) Relative mRNA levels of EMT-TFs in the U-251 cells transfected with Fra1 siRNA and treated with Wnt3a (200 ng/ml) for 48 h. Lef1 served as a negative control; $*P < 0.05$. (I, J) The protein levels of EMT-TFs in the U-251 cells transfected with Fra1 siRNA and treated with Wnt3a (200 ng/ml) for 48 h; $*P < 0.05$.

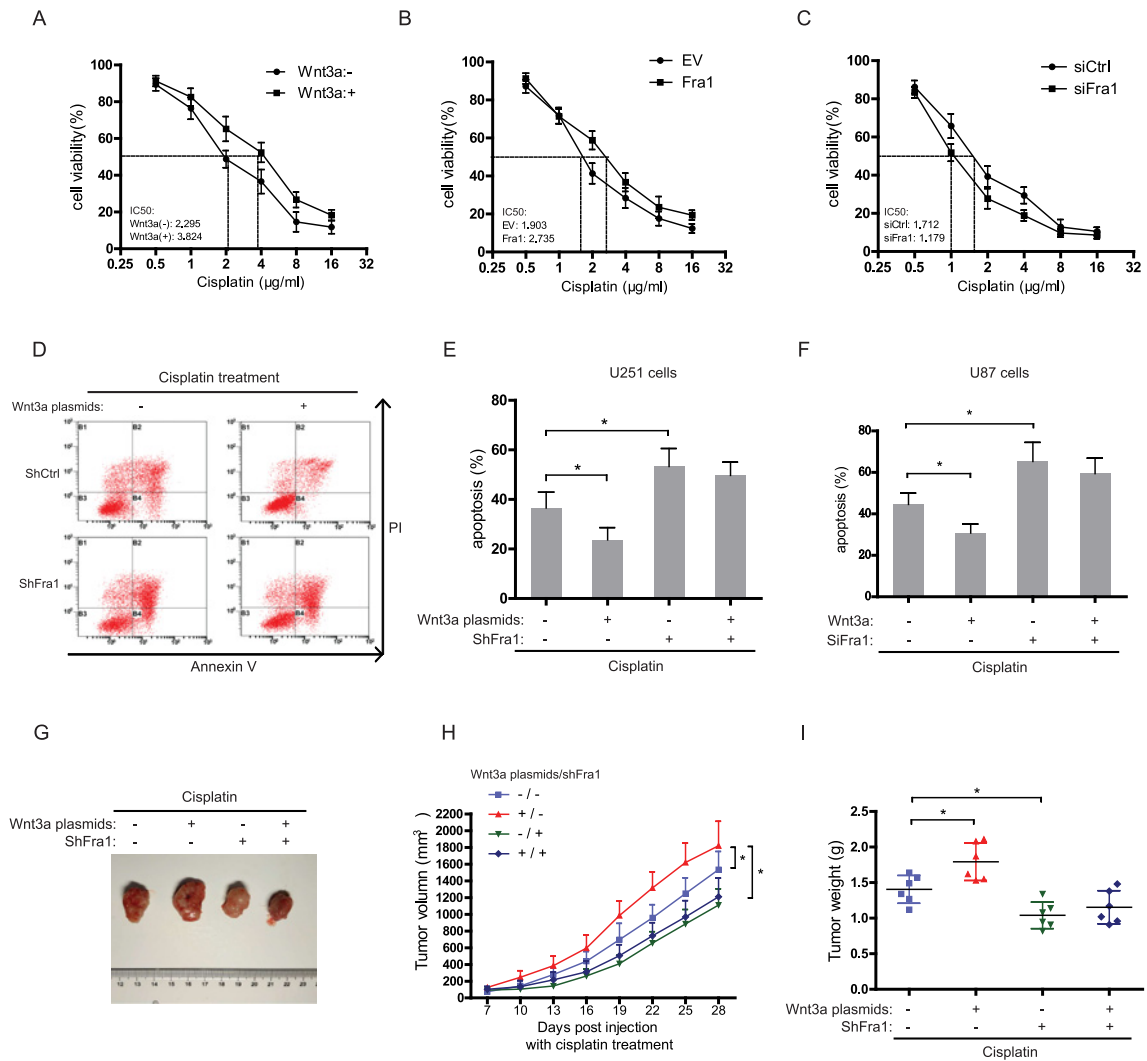


Figure 4. Fra1 determines the cisplatin resistance of glioma cells induced by Wnt/ β -catenin signalling *in vitro* and *in vivo* (A–C) The viability of U-251 cells treated with Wnt3a (200 ng/ml) for 48 h (A) or transfected with Fra1 expression plasmids (B) or siRNA (C) were determined by the CCK-8 assay under different concentrations of cisplatin. (D) Apoptosis levels of the U-251 cells with stable knockdown of Fra1 and overexpression of Wnt3a were measured 48 h after 2 μ g/ml cisplatin addition. (E) Analysis of three separate experiments in (D); * P <0.05. (F) Apoptosis levels of the U-87 cells with Fra1 siRNA transfection and 200 ng/ml Wnt3a treatment were measured 48 h after 2 μ g/ml cisplatin addition; * P <0.05. (G) Representative images of the tumour specimens dissected on day 28. (H) Time course of tumour volume progression, as determined by caliper measurements; * P <0.05. (I) Tumour weights measured 18 days after the beginning of cisplatin treatment; * P <0.05.

invasive capacities in the U-251 cells stimulated with Wnt3a was completely attenuated by Fra1 silencing (Figure 3E,G). Based on Figure 1, the expressions of EMT-TFs were analysed to understand the underlying molecular mechanism. As shown in Figure 3H–J, deprivation of Fra1 significantly disrupted the up-regulation of EMT-TFs induced by Wnt3a. The above findings reveal that up-regulation of Fra1 by Wnt3a is indispensable for the EMT induced by Wnt/ β -catenin signalling in glioma cells.

Fra1 determines the cisplatin resistance of glioma cells induced by Wnt/ β -catenin signalling *in vitro* and *in vivo*

In order to explore the role of Wnt/ β -catenin/Fra1 axis in the chemoresistance of glioma cells, cisplatin resistance was analysed by *in vitro* and *in vivo* experiments. As shown in Figure 4A, the cisplatin resistance of glioma cells with Wnt3a stimulation was 1.67-fold higher than that of the control group, as measured by the IC50 values for cisplatin

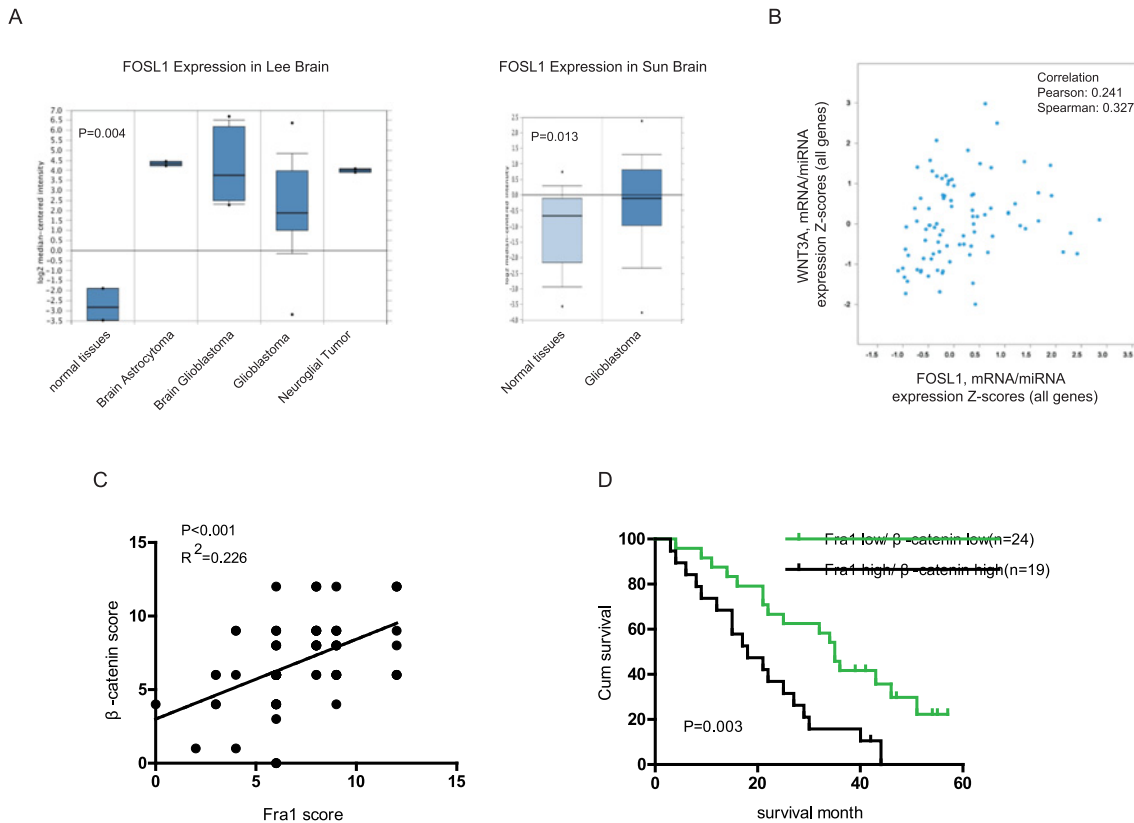


Figure 5. Abnormal Wnt/β-catenin/Fra1 signalling axis indicates a poor prognosis in glioma patients

(A) Investigation of *Fra1* mRNA levels in glioblastoma cancer microarray data sets (Lee Brain and Sun Brain sets) using OncoPrint (www.oncoPrint.org). Higher *Fra1* mRNA levels were found in glioblastoma ($n=78$) when compared with normal tissues ($n=3$) in Lee Brain data set. Higher *Fra1* mRNA levels were found in glioblastoma ($n=81$) compared with normal brain tissues ($n=23$) in Sun Brain data set. (B) Analysis of the TCGA Glioblastoma database (TCGA, Nature 2008) using cBioPortal showing the correlation between *Wnt3a* and *Fra1* mRNA levels. (C) Linear regression between immunostaining intensity of *Fra1* and β-catenin. (D) The association of *Fra1* and β-catenin levels with prognosis of glioma patients. Kaplan–Meier OS curves indicated low expressions of *Fra1* and β-catenin in glioma tissues were significantly associated with a better OS rate. The ‘low’ or ‘high’ of *Fra1* and β-catenin levels was defined according to its cut-off value, which was defined as the median value of the cohort of patients tested; $P < 0.05$ by log-rank test.

over 48 h treatment. Similarly, overexpression of *Fra1* promoted cisplatin resistance, with IC₅₀ increased 1.44-fold as compared with control (Figure 4B). However, knockdown of *Fra1* sensitized glioma cells to cisplatin, with IC₅₀ decreased to 69 percent (Figure 4C). Furthermore, the U-251 cells with stable *Fra1* knockdown and *Wnt3a* overexpression were constructed and examined for cisplatin sensitivity. *Wnt/β-catenin* signalling activation decreased cisplatin-induced apoptosis in U-251 cells compared with control but failed to protect from apoptosis after *Fra1* knockdown (Figure 4D,E), which was also observed in the U-87 cells with *Fra1* siRNA transfection and *Wnt3a* treatment (Figure 4F). Xenograft tumour assay was taken to test whether such an effect would occur *in vivo*. The results showed that overexpressed *Wnt3a* increased resistance to cisplatin, and knockdown of *Fra1* sensitized glioma cells to cisplatin. Notably, silence of *Fra1* inhibited the protective effect of *Wnt3a* against cisplatin (Figure 4G–I). In summary, *Wnt/β-catenin/Fra1* axis displays a critical role in the cisplatin resistance of glioma cells.

Abnormal Wnt/β-catenin/Fra1 signalling axis indicates a poor prognosis in glioma patients

Through analysing glioblastoma cancer microarray data sets on OncoPrint, a higher *Fra1* mRNA level was found in glioblastoma when compared with normal brain tissue (Figure 5A). Besides, the data from TCGA database (Glioblastoma: TCGA, Nature 2008 [20]) were analysed to determine the correlation between *Wnt3a* and *Fra1* at mRNA level

Table 1 Correlation of the expression of Fra1 and β -catenin with clinicopathological features in glioma

	Total	Fra1		P	β -catenin		P
		Low	High		Low	High	
Gender	87	52	35	0.788	49	38	0.113
Male	51	31	20		26	25	
Female	36	21	15	0.465	23	13	0.148
Age							
<40	17	10	7	0.678	8	9	0.326
\geq 40	70	42	28		41	29	
Tumour location				0.621			0.562
Frontal	23	13	10		12	11	
Parietal	21	12	9		15	6	
Occipital	17	11	6		9	8	
Temporal	21	13	8		10	11	
Unknown	5	3	2	3	2		
Tumour diameter				0.003			0.001
<4cm	38	24	14		21	17	
\geq 4cm	49	28	21	28	21		
WHO grade							
II	43	34	9		31	13	
III	27	16	11		18	9	
IV	17	2	15		1	16	

in clinical glioma specimens by the cBioPortal platform and a positive correlation was observed (Figure 5B). Immunohistochemical staining was implemented to analyse the clinical relevance of Fra1 and β -catenin protein levels in 87 human glioma specimens. As shown in Table 1, the β -catenin and Fra1 staining were positively correlated with WHO grade ($P < 0.05$) but not with gender, age, tumour location, tumour diameter ($P > 0.05$). Moreover, the Fra1 expression levels paralleled with the changes of β -catenin in the glioma cases as shown by immunohistochemical analysis ($P < 0.05$) (Figure 5C). The 3-year overall survival (OS) rate of the Fra1 and β -catenin double-high expression group was significantly lower than that of the Fra1 and β -catenin double-low expression group (15.8% compared with 45.8%, $P = 0.003$) (Figure 5D). These data suggest that aberrant activation of Wnt/ β -catenin/Fra1 signalling axis drives glioma progression and indicates a poor prognosis.

Discussion

As the canonical Wnt pathway, β -catenin-dependent pathway plays an important role in many biological progresses such as tissue homeostasis, embryonic development and tumorigenesis. In recent studies, it has been reported that aberrant activation of Wnt/ β -catenin signalling contributed to glioma development [21]. The evidence based on clinical glioma specimens also showed the up-regulation of β -catenin and Tcf-4 in cancer tissues compared with normal brain [22]. The siRNA induced knockdown of Lef1 or β -catenin suppressed cell proliferation and invasion as well as promoting apoptosis in glioma cells [23,24]. Besides, various target genes result in the multifunction of Wnt/ β -catenin signalling in cancer development. The high invasive ability of glioma cancer to degrade extracellular matrix (ECM) depends on MMPs, which are the crucial genes regulated by Wnt signalling [25]. Other target genes including *cyclinD1* and *c-myc* are required for cellular proliferation in glioma [26]. Here, we found that the EMT-TF, Fra1, which were transactivated by β -catenin/Tcf/Lef complex, promoted the EMT programme induced by Wnt/ β -catenin signalling in glioma cells. Knockdown of Fra1 decreased Wnt3a-induced EMT markers as well as migration and invasion of glioma cells, which suggested Fra1 as a key factor to form a cross-talk between Wnt pathway and EMT transition. Interestingly, we observed that Wnt/ β -catenin/Fra1 axis conferred the cisplatin resistance of glioma *in vitro* and *in vivo*. Further analysis of clinical specimens revealed that aberrant expression of Fra1 or β -catenin in glioma tissues indicated a poor prognosis.

The activator protein-1 complex (AP1) consisting of homo- or heterodimers of Fos, Jun, ATF and MAF family members has been implicated as central activator of tumour cell invasion. Among them, Fra1 is one of the most frequently aberrant expressed AP1 proteins in a variety of tumour types including glioma [27-29]. In previous studies, Fra1 was explored as a key EMT switch and its ability to promote migration and invasion was found in many cancer

types [30,31]. In addition, up-regulation of Fra1 mediates the resistance to anticancer drug nimustine (ACNU) in glioma cells [32]. More and more target genes of Fra1 have been demonstrated to explain the roles of the important regulator in biological processes. The EMT-TFs such as Snail, Slug and Zeb1 along with the EMT markers including Vimentin, MMP2 and MMP9 are the classical genes regulated by Fra1 [12]. Interestingly, some Fra1-induced factors like Zeb1 and MMPs could also be regulated by β -catenin, which indicated that Fra1 might serve as a node factor in Wnt/ β -catenin pathway [33,34]. In the present work, we observed that activating Wnt/ β -catenin pathway increased EMT-TFs, whereas knockdown of Fra1 reversed the effect in glioma cells. However, other proteins involved in Wnt/ β -catenin/Fra1 axis deserve to be investigated in the future.

In studies with GBM-derived cell lines, activation of the canonical Wnt/ β -catenin pathway could modulate a set of EMT-TFs including Twist1 and Zeb1/2 [8]. Twist1 and Zeb1/2 are the cardinal activators of EMT in glioma, which have been well studied. Initial reports demonstrated that Twist1 was overexpressed in MG and enhanced GBM invasion [35]. Knockdown of Twist1 impaired the capacities of migration and invasion as well as sphere formation in GBM [9]. Examination of specimens derived from glioblastoma patients showed significantly higher Zeb2 level with early relapse and fast tumour progression [36]. In our study, the inductions of Twist1 and Zeb1/2 by activating Wnt/ β -catenin signalling were also observed. However, by comparison, alteration of Fra1 expression was the most remarkable among these EMT-TFs. Interestingly, aberrant expressions of β -catenin and Fra1 were predominant at the invasive front of the tumour [8,37]. Therefore, the roles of Wnt/ β -catenin/Fra1 pathway in activating the growth of tumour bud need further investigations.

In conclusion, our findings demonstrate that Wnt/ β -catenin signalling can induce the EMT programme of glioma cells by up-regulating Fra1 expression. Further results reveal that Wnt/ β -catenin signalling activates the transcription of Fra1 by directly binding β -catenin/Tcf/Lef transcriptional complex to the WRE located at the proximal region of Fra1 promoter. Phenotype experiments and clinical analysis support the critical role of Fra1 in mediating the glioma aggressiveness induced by Wnt/ β -catenin signalling. Therefore, the correlation between Wnt/ β -catenin signalling and Fra1 raises the possibility of developing novel biomarkers and therapeutic targets for glioma.

Funding

This work was supported by the National Natural Science Foundation of China (81401789) and Hebei medical science research program (ZL20140183).

Competing interests

The authors declare that there are no competing interests associated with the manuscript.

Author contribution

H.L. conceived and designed the study. L.Z., H.L. and X.M. performed the experiments and wrote the manuscript. L.Z., J.C. and Z.P. analysed and interpreted the data. L.Z., H.L., X.M., J.C. and Z.P. contributed reagents and materials and helped to draft the manuscript. All authors read and approved the final manuscript.

Abbreviations

AP1, activator protein-1 complex; CCK-8, cell counting kit-8; EMT, epithelial-mesenchymal transition; EMT-TF, EMT-related transcription factors; Fra1, Fos-related antigen-1; IC50, half maximal inhibitory concentration; IHC, Immunohistochemistry; LiCl, lithium chloride; MG, malignant glioma; MMP, matrix metalloproteinase; OS, overall survival.

References

- 1 Malzkorn, B. and Reifenberger, G. (2016) Practical implications of integrated glioma classification according to the World Health Organization classification of tumors of the central nervous system 2016. *Curr. Opin. Oncol.* **28**, 494–501
- 2 Reardon, D.A., Galanis, E., DeGroot, J.F., Cloughesy, T.F., Wefel, J.S., Lamborn, K.R. et al. (2011) Clinical trial end points for high-grade glioma: the evolving landscape. *Neuro-Oncol.* **13**, 353–361
- 3 Nie, E., Jin, X., Wu, W., Yu, T., Zhou, X., Zhi, T. et al. (2016) BACH1 promotes temozolomide resistance in glioblastoma through antagonizing the function of p53. *Sci. Rep.* **6**, 39743
- 4 Iwadate, Y. (2016) Epithelial-mesenchymal transition in glioblastoma progression. *Oncology Letters* **11**, 1615–1620
- 5 Kahlert, U.D., Nikkhah, G. and Maciaczyk, J. (2013) Epithelial-to-mesenchymal(-like) transition as a relevant molecular event in malignant gliomas. *Cancer Lett.* **331**, 131–138
- 6 Yang, H.W., Menon, L.G., Black, P.M., Carroll, R.S. and Johnson, M.D. (2010) SNAI2/slug promotes growth and invasion in human gliomas. *BMC Cancer* **10**, 301
- 7 Han, S.P., Kim, J.H., Han, M.E., Sim, H.E., Kim, K.S., Yoon, S. et al. (2011) SNAI1 is involved in the proliferation and migration of glioblastoma cells. *Cell. Mol. Neurobiol.* **31**, 489–496

- 8 Kahlert, U.D., Maciaczyk, D., Doostkam, S., Orr, B.A., Simons, B., Bogiel, T. et al. (2012) Activation of canonical WNT/ β -catenin signaling enhances *in vitro* motility of glioblastoma cells by activation of ZEB1 and other activators of epithelial-to-mesenchymal transition. *Cancer Lett.* **325**, 42–53
- 9 Mikheeva, S.A., Mikheev, A.M., Petit, A., Beyer, R., Oxford, R.G., Khorasani, L. et al. (2010) TWIST1 promotes invasion through mesenchymal change in human glioblastoma. *Mol. Cancer* **9**, 194
- 10 Zhang, X., Wu, J., Luo, S., Lechler, T. and Zhang, J.Y. (2016) FRA1 promotes squamous cell carcinoma growth and metastasis through distinct AKT and c-Jun dependent mechanisms. *Oncotarget* **7**, 34371–34383
- 11 Lombaerts, M., van Wezel, T., Philippo, K., Dierssen, J.W., Zimmerman, R.M., Oosting, J. et al. (2006) E-cadherin transcriptional downregulation by promoter methylation but not mutation is related to epithelial-to-mesenchymal transition in breast cancer cell lines. *Br. J. Cancer* **94**, 661–671
- 12 Caramel, J., Papadogeorgakis, E., Hill, L., Browne, G.J., Richard, G., Wierinckx, A. et al. (2013) A switch in the expression of embryonic EMT-inducers drives the development of malignant melanoma. *Cancer Cell* **24**, 466–480
- 13 Diesch, J., Sanij, E., Gilan, O., Love, C., Tran, H., Fleming, N.I. et al. (2014) Widespread FRA1-dependent control of mesenchymal transdifferentiation programs in colorectal cancer cells. *PLoS ONE* **9**, e88950
- 14 Anastas, J.N. and Moon, R.T. (2013) WNT signalling pathways as therapeutic targets in cancer. *Nat. Rev. Cancer* **13**, 11–26
- 15 Nager, M., Bhardwaj, D., Canti, C., Medina, L., Noguees, P. and Herreros, J. (2012) β -catenin signalling in glioblastoma multiforme and glioma-initiating cells. *Chemother. Res. Pract.* **2012**, 192362
- 16 Liu, Y., Yan, W., Zhang, W., Chen, L., You, G., Bao, Z. et al. (2012) MiR-218 reverses high invasiveness of glioblastoma cells by targeting the oncogenic transcription factor LEF1. *Oncol. Rep.* **28**, 1013–1021
- 17 Yue, X., Cao, D., Lan, F., Pan, Q., Xia, T. and Yu, H. (2016) MiR-301a is activated by the Wnt/ β -catenin pathway and promotes glioma cell invasion by suppressing SEPT7. *Neuro-Oncol.* **18**, 1288–1296
- 18 Lui, H., Lui, Z., Jiang, B., Peng, R., Ma, Z. and Lu, J. (2015) Sox9 overexpression promotes glioma metastasis via Wnt/ β -catenin signaling. *Cell Biochemistry and Biophysics* **73**, 205–212
- 19 Sun, J. and Jin, T. (2008) Both Wnt and mTOR signaling pathways are involved in insulin-stimulated proto-oncogene expression in intestinal cells. *Cell. Signal.* **20**, 219–229
- 20 Cancer Genome Atlas Research Network (2008) Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* **455**, 1061–1068
- 21 Paw, I., Carpenter, R.C., Watabe, K., Debinski, W. and Lo, H.W. (2015) Mechanisms regulating glioma invasion. *Cancer Lett.* **362**, 1–7
- 22 Zhang, J., Huang, K., Shi, Z., Zou, J., Wang, Y., Jia, Z. et al. (2011) High β -catenin/Tcf-4 activity confers glioma progression via direct regulation of AKT2 gene expression. *Neuro-Oncol.* **13**, 600–609
- 23 Gao, X., Mi, Y., Ma, Y. and Jin, W. (2014) LEF1 regulates glioblastoma cell proliferation, migration, invasion, and cancer stem-like cell self-renewal. *Tumour Biol.* **35**, 11505–11511
- 24 Pu, P., Zhang, Z., Kang, C., Jiang, R., Jia, Z., Wang, G. et al. (2009) Downregulation of Wnt2 and beta-catenin by siRNA suppresses malignant glioma cell growth. *Cancer Gene Ther.* **16**, 351–361
- 25 Kamino, M., Kishida, M., Kibe, T., Ikoma, K., Iijima, M., Hirano, H. et al. (2011) Wnt-5a signaling is correlated with infiltrative activity in human glioma by inducing cellular migration and MMP-2. *Cancer Sci.* **102**, 540–548
- 26 Davidson, G. and Niehrs, C. (2010) Emerging links between CDK cell cycle regulators and Wnt signaling. *Trends Cell Biol.* **20**, 453–460
- 27 Galvagni, F., Orlandini, M. and Oliviero, S. (2013) Role of the AP-1 transcription factor FOSL1 in endothelial cells adhesion and migration. *Cell Adh. Migr.* **7**, 408–411
- 28 Belguise, K., Kersual, N., Galtier, F. and Chalbos, D. (2005) FRA-1 expression level regulates proliferation and invasiveness of breast cancer cells. *Oncogene* **24**, 1434–1444
- 29 Debinski, W. and Gibo, D.M. (2005) Fos-related antigen 1 modulates malignant features of glioma cells. *Mol. Cancer Res.* **3**, 237–249
- 30 Sayan, A.E., Stanford, R., Vickery, R., Grigorenko, E., Diesch, J., Kulbicki, K. et al. (2012) Fra-1 controls motility of bladder cancer cells via transcriptional upregulation of the receptor tyrosine kinase AXL. *Oncogene* **31**, 1493–1503
- 31 Tkach, V., Tulchinsky, E., Lukanidin, E., Vinson, C., Bock, E. and Berezin, V. (2003) Role of the Fos family members, c-Fos, Fra-1 and Fra-2, in the regulation of cell motility. *Oncogene* **22**, 5045–5054
- 32 Meise, R., Tomicic, M.T., Kaina, B. and Christmann, M. (2012) The chloroethylating anticancer drug ACNU induces FRA1 that is involved in drug resistance of glioma cells. *Biochim. Biophys. Acta* **1823**, 1199–1207
- 33 Wu, D.W., Lin, P.L., Cheng, Y.W., Huang, C.C., Wang, L. and Lee, H. (2016) DDX3 enhances oncogenic KRAS-induced tumor invasion in colorectal cancer via the β -catenin/ZEB1 axis. *Oncotarget* **7**, 22687–22699
- 34 Sui, H., Xu, H., Ji, Q., Liu, X., Zhou, L., Song, H. et al. (2015) 5-hydroxytryptamine receptor (5-HT1DR) promotes colorectal cancer metastasis by regulating Axin1/ β -catenin/MMP-7 signaling pathway. *Oncotarget* **6**, 25975–25987
- 35 Rahme, G.J. and Israel, M.A. (2015) Id4 suppresses MMP2-mediated invasion of glioblastoma-derived cells by direct inactivation of Twist1 function. *Oncogene* **34**, 53–62
- 36 Qi, S., Song, Y., Peng, Y., Wang, H., Long, H., Yu, X. et al. (2012) ZEB2 mediates multiple pathways regulating cell proliferation, migration, invasion, and apoptosis in glioma. *PLoS ONE* **7**, e38842
- 37 Pereira, C.H., Morais, M.O., Martins, A.F., Soares, M.Q., Alencar Rde, C., Batista, A.C. et al. (2016) Expression of adhesion proteins (E-cadherin and β -catenin) and cell proliferation (Ki-67) at the invasive tumor front in conventional oral squamous cell and basaloid squamous cell carcinomas. *Arch. Oral Biol.* **61**, 8–15