Chinese-German J Clin Oncol
DOI 10.1007/s10330-314-0013-9
December 2014, Vol. 13, No. 12, P594–P599

Expression of FOXG1 is associated with the malignancy of human glioma

Zhiwei Shao[1], Beibei Cong[2], Aihua Sui[3], Kai Meng[1], Yihe Dou (✉)[1]

[1] Department of Neurosurgery, the Affiliated Hospital of Qingdao University Medical College, Qingdao University, Qingdao 266000, China
[2] Department of Immunological Laboratory, Qiingdao University School of Medicine, Qingdao University, Qingdao 266003, China
[3] Department of The Central Liboratology, the Affiliated Hospital of Qingdao University Medical College, Qingdao University, Qingdao 266003, China

Received: 1 October 2014 / Revised: 30 October 2014 / Accepted: 25 November 2014

Abstract *Objective:* Recent evidence indicates that the increased expression of FOXG1 is associated with tumor genesis. This study was designed to explore the expression and role which FOXG1 plays in human glioma. *Methods:* We detected the expression of FOXG1 by immunohistochemistry in glioma tissue samples. Following the down-regulation of FOXG1 in glioma cell lines by a specific short hairpin RNA, the function of FOXG1 in proliferation and apoptosis was assessed. *Results:* Glioma tissues exhibited notably higher expression of FOXG1 compared with control brain tissues and was positively correlated with histological malignancy. The down-regulation of FOXG1 in glioma cells led to a cell apoptosis *in vitro*. *Conclusion:* The overexpression of FOXG1 is a novel glioma malignancy marker, and FOXG1 may be used as a new target in therapeutic strategies for human glioma.

Key words FOXG1; glioma; expression; apoptosis

Glioma is one of the most aggressive tumors and is exceptionally difficult to treat. Although glioma therapies have improved significantly, the outcome of patients remains poor. Glioblastoma (GBM; World Health Organization grade IV glioma) is the most malignant and frequent primary brain cancer, representing up to 60% of all astrocyte-lineage tumors. GBM patients have a median survival time ranging from 9 to 15 months because the aggressive and recurring nature of this cancer can only be temporarily contained by surgical resection followed by combined radiotherapy and chemotherapy [1–3]. Given the fatal nature of malignant gliomas and the fact that very little is known about their etiological factors, the potential role of regulating proteins in glioma genesis warrants further investigation.

Recent reports indicate that development of tumor is often associated with regulating of a variety of transcription factors. FOXG1, also known as brain factor-1, is a member of Forkhead box family of transcription factors [4–5]. Recent studies showed that FOXG1 was upregulated and inversely associated with the expression levels of p21WAF1/CIP1 in ovarian cancer [6]. The overexpression of FOXG1 was significantly correlated with high-grade ovarian cancer. Immunohistochemical analysis on ovarian cancer tissue array was further confirmed that FOXG1 was highly expressed and significantly correlated with high-grade ovarian cancer. FOXG1 contains a highly conserved DNA-binding domain, which binds to specific DNA sequences and regulates gene expression. Paper also reported that FOXG1 overexpression may contribute to the maintenance of the undifferentiated state in hepatoblastomas and could be a potential target for molecular therapeutics [7–10]. Furthermore, excess FOXG1 expression *in vivo* is associated with neural progenitor cell overgrowth; an effect requiring DNA-binding and repressor activity. Foxg1 acts to maintain the normal neural stem/progenitor cells (NSPC) state at the expense of neural cell differentiation and its inactivation causes a dramatic perturbation of cerebral cortex development as a result of premature NSPC differentiation. The FOXG1 protein acts, at least in part, by forming transcription repression complexes with other regulating proteins. In this study we sought to characterize the expression and function of FOXG1 in glioma cells [11–15].

Correspondence to: Yihe Dou. Email: 50202524@163.com

To understand the requirement of FOXG1 in gliomas, we firstly compared the FOXG1 expression of low-grade and high-grade gliomas (LGG and HGG) in brain tumor surgery patients. Then a glioma cell line was used to study the FOXG1 function in glioma cells proliferation and apoptosis. Our research may provide an effective histology method for glioma detection and an attractive strategy for glioma therapy.

Materials and methods

Human glioma specimens and cell lines

All the 58 specimens, with detailed clinical database, were gained from the glioma patients in the Neurosurgery Department of Affiliated Hospital of Qingdao University during January to June 2014. None of the patients had received chemotherapy or radiotherapy. The specimens, gained on the operating table, were fixed in 10% neutral formalin and desiccated and embedded in paraffin for immunohistochemistry.

Human glioblastoma cell lines U118 were purchased from the Cell Bank of the Shanghai Branch of Chinese Academy of Sciences. The human normal glial cell line HEB was purchased from Zhongshan School of Medicine (China)

Immunohistochemistry

The paraffin-embedded human pancreatic resection slides were stained for FOXG1 expression. Paraffin was removed by heating sections at 60 ℃ for 2 h, followed by incubations in 100% xylene for 2 min each. Subsequent incubations were in 100%, 95%, 80% and 75% ethanol for 4 min, respectively. Specimens were then microwaved in 1 litre of a sodium citrate buffer (pH 6) for 45 min for heat-mediated antigen retrieval. A 1% solution of hydrogen peroxide in methanol was used to block endogenous peroxidase activity before transferring the sections into phosphate-buffered saline (PBS) (0.1 M; pH 7.2). The slides were incubated at 37 ℃ temperature for 62 min against anti-FOXG1 primary antibody with appropriate dilution, followed by 4 washes in PBS. After 37℃ temperature for 20 min incubation of dilute secondary antibody, visualization was achieved with DAB reagent following the manufacturer's protocol. The positive reaction of FOXG1 protein is visible as brown particles in the nucleus. The intensity of staining was scored as – (negative), + (moderate), ++ (strong) and +++ (marked).

Reverse transcription-PCR

Total cellular RNAs were extracted using Trizol reagent (Invitrogen, USA) following the manufactures' instructions. Two micrograms of total RNA was used as a template for cDNA synthesis using first strand cDNA synthesis kit. Primers designed for FOXG1 were 5'-CCTGCTGGCTCAGAAATGC-3' as forward and 5'-GAG GCGAGGCACTACTTCC-3' as reverse, primers 5'-TG GAACGGTGAAGGTGACAG-3' and 5'-GGCTTTTAG GATGGCAAGGG-3' were for detection of â-actin as internal control. The band densities were calculated using Quantity One.

Cell proliferation assay

Cell proliferation was tested by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, USA). Glioblastoma cells U118 were plated in 96-well microplte formats according to the manufacturer's recommendation. Cell lines were seeded in five replication wells at 5000 viable cells per well and cultured for 0 h, 6 h, 12 h, 24 h, 48 h and 72 h. Following MTT uptake over a period of 4 h, cells were lysed in 150 μL of dimethylsulfoxide and absorbance was measured using a fluorescence microplate reader.

Western blot analysis

Cell were washed with ice-cold phosphate-buffered saline (PBS) three times. Then cells were then solubilized in 500 μL of lysis buffer and 5 μL of PMSF (Beyotime, China). Samples were centrifuged at 12,000 rpm for 5 min at 4 ℃ to separate the membrane fraction from the cytosolic fraction. After boiling for 5 min, equivalent amounts of protein (30 μg) were resolved by 12% SDS-PAGE, electroblotted onto PVDF membrane and immunoreacted overnight with FOXG1 (Abcam, UK) antibody, followed by 2 h incubation with the secondary antibody (Bioss, CHN) conjugated with HRP. Chemiluminescent signals were generated by SuperSignal West Pico Trial Kit (Thermo, USA) and detected on Vilber Lourmat.

Apoptosis detection by flow cytometry

After 24 h transfection, the cells were harvested, centrifuged and washed with PBS for three times. A total of 100 μL incubation media supplemented with 5% FBS was added to resuspended cells in 1.5 mL centrifuge tube. The slides were incubated at room temperature for 20 min without light with 100 μL Guava Nexin Reagant. Samples were analyzed by Guava Easy Cyte Mini Flow cytometry instrument, and each sample repeated for 3 times.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed by using the Student *t* test. $P < 0.05$ was considered as significant difference.

Results

Expression of FOXG1 in different histological types of gliomas

We performed immunohistochemical staining of the

Table 1 Tumor FOXG1 positivity by histology using IHC

Groups	Samples	–	+	++	+++	*F*	*P*
Nontumorous	8	8	0	0	0	83.538	0.000
WHO I	8	2	6	0	0		
WHO II	14	0	12	2	0		
WHO III	10	0	0	8	2		
WHO IV	18	0	0	4	14		
Total	58	10	18	14	16		

Table 2 High level of FOXG1 is associated with the grade of human gliomas

Characteristics	High levels of FOXG1 (++/+++)	Low levels of FOXG1 (–/+)	*P*
Age (years)			0.729
Median	48	48	
Range	9–77	13–77	
Sex			0.359
Male	79	92	
Female	31	27	
Grade			0.0001*
I	0	8	
II	2	12	
III	10	0	
IV	18	0	

* Fisher's exact test was used to measure statistical significance. All other data were analyzed by Student's *t* test

58 paraffin-embedded sections obtained from glioma patients using an antibody specific for the FOXG1 protein to determine whether FOXG1 expression was different in the sections. The expression of FOXG1 was detected by IHC in 50 glioma and 8 control tissues. We observed immunoreactivity of FOXG1 protein in the nucleolus (Fig. 1), and a very low level of FOXG1 expression was observed in control tissues compared with glioma tissues. There was a statistically significant difference in FOXG1 expression between glioma samples and nontumorous samples ($P < 0.01$). We observed positive staining of FOXG1 in the nucleolus of glioma tissues of different grades (Fig. 1). In total, 30 of the 50 glioma tissues expressed a high level (++/+++) of FOXG1 (30/50, 60%). In accordance with previous reports, patients with FOXG1 high expression exhibited more aggressive features. The level of FOXG1 was 0% (0/6) in grade I, 14.28% (2/14) in grade II, 100% (10/10) in grade III, and 100% (10/10) in grade IV (Table 1). These differences were significant. However, we did not observe relationships between the expression of FOXG1 with other clinical characteristics, including age and sex (Table 2).

We detected high FOXG1 immunoreactivity (++/+++) in 2 low-grade (LGG, grade I and II) and 28 high-grade (HGG, grade III and IV) gliomas, but not in the control non-tumor brain tissue specimen. The overall high positivity of HGG samples was 100%, while low-grade tumors and non-tumor brain tissue were 8.3% and 0% positive, respectively. There was a significantly higher level of FOXG1 positivity in tumor cells associated with high-grade gliomas when compared to the lower grade tumors ($P < 0.05$).

Disruption of FOXG1 by RNAi inhibits GBM cell proliferation

Previous studies have demonstrated that FOXG1 might increase the malignancy of many kinds of tumor cells. In order to study the FOXG1 function in glioma cells, firstly we detect the FOXG1 expression in glioma cell line U118. U118 cell had strong basal FOXG1 expression in both mRNA and protein level compared to normal human glial cell line HEB (Fig. 2). The rapid proliferation is one feature of tumor cell. To study if FOXG1 play an

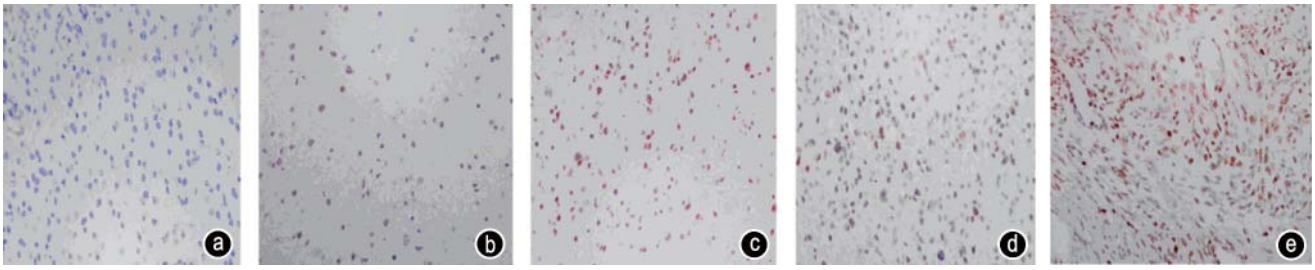

Fig. 1 Immunohistochemical detection of FOXG1 expression in human glioma samples by histology (× 400). (a) Immunohistochemical stain of a non-tumor brain tissue with an antibody against the FOXG1 protein; (b) Immunohistochemical stain of a WHO I glioma section with an antibody against the FOXG1 protein; (c) Immunohistochemical stain of a WHO II glioma section with an antibody against the FOXG1 protein; (d) Immunohistochemical stain of a WHO III glioma section with an antibody against the FOXG1 protein; (e) Immunohistochemical stain of a WHO IV glioma section with an antibody against the FOXG1 protein

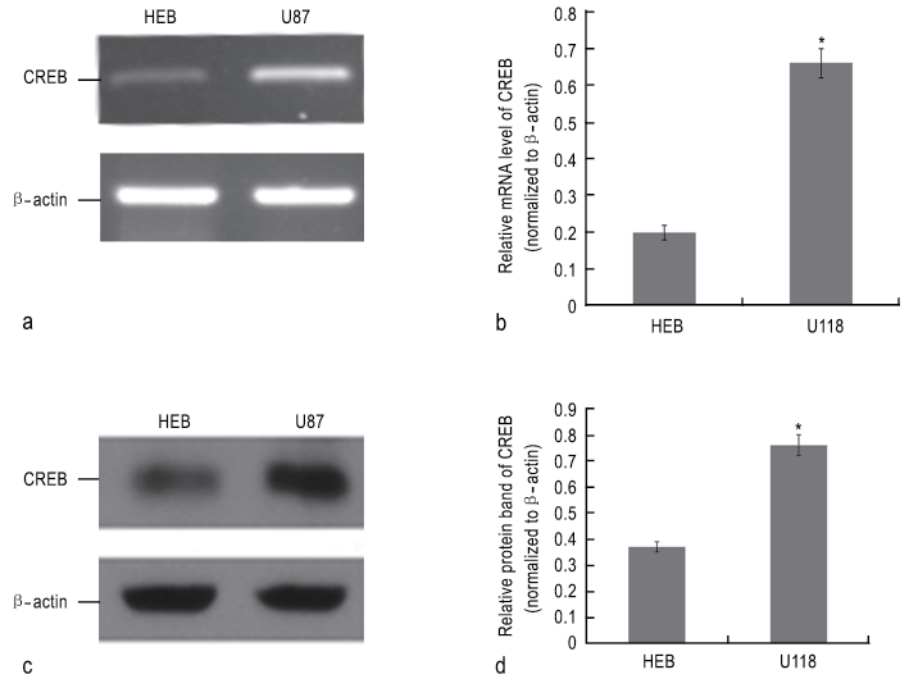

Fig. 2 FOXG1 expression in U118 glioma cell lines and normal human glial HEB cells. (a) RT-PCR detection of FOXG1 expression in HEB and U118 cell lines; (b) Relative mRNA levels of FOXG1 in HEB and U118 cell lines; (c) Western blot detection of FOXG1 expression in in HEB and U118 cell lines. (d) Relative protein levels of FOXG1 in HEB and U118 cell lines. * $P < 0.05$ vs. HEB

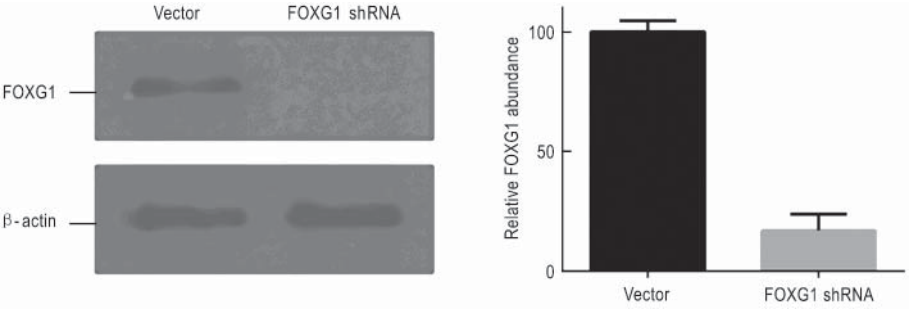

Fig. 3 Western blot analysis demonstrating interference of FOXG1 approaching 80% compared with control cells

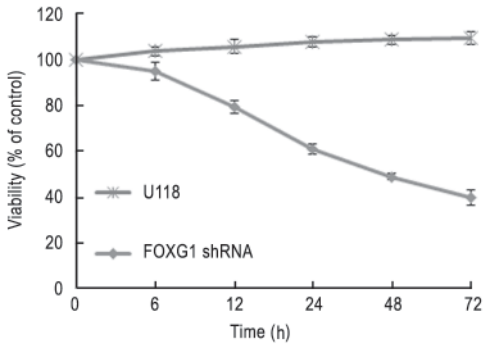

Fig. 4 Cell viability analysis of shFOXG1 in U118 cells. FOXG1 interference reduces U118 cells proliferation significantly. U118 cells expression nontargeting shRNA or FOXG1 shRNA were detected by MTT assay for 6 h to 72 h. Knocking down FOXG1 expression reduces tumor cell proliferation significantly

important role in U118 cells' proliferation, we use RNA interference (RNAi) to disrupt FOXG1 gene expression. We use a lentivirus vectors with shRNA sequences (TCTGTCCCTCAACAAGTGC) targeting FOXG1 and non-targeting shRNA sequence as control. We analyzed FOXG1 expression in U118 cells transduced with FOXG1 shRNA lentivirus by Western blot analysis and observed a significant inhibition of FOXG1 expression (–80%) in FOXG1 shRNA-transduced cells, compared with control cells (Fig. 3).

We next studied the effects of FOXG1 down-regulation in proliferation of U118 cells. MTT assay showed that U118 cell proliferation was significantly depressed in FOXG1 interference U118 cells (Fig. 4). These results indicate that FOXG1 protein may be involved in the regulation of cell proliferation and apoptosis pathway. In order to confirm the FOXG1 function in glioma cells apoptosis. An annexin V/7-AAD staining flow cytometry was carried out to evaluate the apoptotic rate. Fig. 5 shows that the apoptotic rate was significantly elevated in shFOXG1 U118 cells in 24 h (20.7%) and 48 h (45.5%) compared to

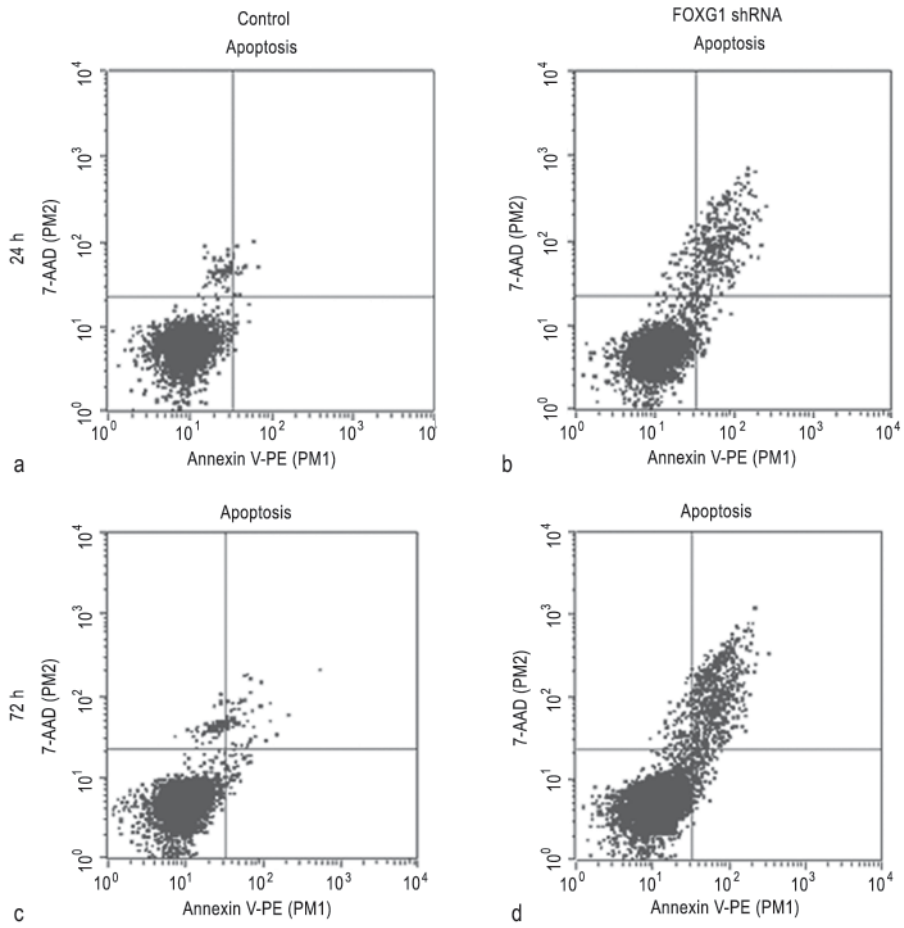

Fig. 5 Apoptosis measured by Annexin V/7-AAD staining flow cytometry following FOXG1 interference as indicated time in U118 cells. FOXG1 interference evoked U118 cells apoptosis in 24 h and 72 h compared to nontargeting shRNA. Events in each of the four quadrants are as follows: (a) Mostly nuclear debris; (b) Cells in the late stages of apoptosis; (c) Viable cells; (d) Cells in the early to mid-stages of apoptosis

control vector group (1.1% and 2.1%). Therefore, our results suggest that FOXG1 down-regulation in U118 cells inhibits proliferation and survival and induces apoptosis *in vitro*.

Discussion

Gliomas are the most common brain tumors with high morbidity and mortality. In the past several decades, the prognosis for malignant gliomas has not significantly improved. Median survival of patients with GBM is usually less than 1 year from the time of diagnosis, and most patients die within 2 years even in the most favorable circumstances. One important reason for treatment failure is the uncontrollable invasion and migration of glioma cells, which ultimately leads to diffuse growth and recurrence of the tumor. Therefore, new diagnostics strategies of glioma cells are urgently required [2, 8–9, 16].

FOXG1 is highly expressed in the telencephalon and has a crucial role in both proliferation and differentiation of neocortical progenitors during brain development. Indeed, v-Qin and c-Qin, the orthologs of FOXG1, have been reported to induce oncogenic transformation of chicken embryo fibroblasts, suggesting FOXG1 may function as an oncogene in human cancer. FOXG1 overexpression is known to cause overgrowth by counteracting signaling induced by cytostatic factors such as TGF-β and BMP4 through the repression of the transcription of cyclin-dependent kinase inhibitors p15Ink4b and p21cip1, as well as by reducing the frequency of normal programmed cell death or apoptosis [4, 6, 10–12].

In this study, we showed that the FOXG1 are abundantly present in human glioma specimens and cell lines. We also showed that the FOXG1 staining patterns varied by histology. Therefore, we conclude that high levels of FOXG1 are correlated with the progression of glioma.

Because FOXG1 is highly expressed in glioma cells, we deduced that it should contribute to the malignancy of glioma. RNAi is a biological process in which RNA molecules inhibit gene expression, typically by causing the destruction of specific mRNA molecules. Here we found that FOXG1 interference inhibited glioma cell U118 proliferation and triggered cell apoptosis in time-depend manner.

In summary, our results show that the highly expressed FOXG1 transcription factor is highly expressed in glioma

and associated with histological types of gliomas. Our findings also suggest that high expression of FOXG1 may promote glioma cell proliferation and survival. Targeting FOXG1 might be an attractive strategy for the detection and therapy of pancreatic carcinomas.

Conflicts of interest

The authors indicated no potential conflicts of interest.

References

1. Liu C, Sage J C, Miller M R, *et al.* Mosaic analysis with double markers reveals tumor cell of origin in glioma. Cell, 2011, 146: 209–221.
2. Li Y, Wang D, Wang L, *et al.* Distinct genomic aberrations between low-grade and high-grade gliomas of Chinese patients. PLoS One, 2013, 8: e57168.
3. Ghany DA. Impact of adjuvant chemotherapy delay on survival in cancer breast patients. Chinese-German J Clin Oncol, 2013, 12: 20–24.
4. Verginelli F, Perin A, Dali R, *et al*. Transcription factors FOXG1 and Groucho/TLE promote glioblastoma growth. Nat Commun, 2013, 4: 2956.
5. Chen J, Li Y, Yu TS, *et al.* A restricted cell population propagates glioblastoma growth after chemotherapy. Nature, 2012, 488: 522–526.
6. Chan DW, Liu VW, To RM, *et al.* Overexpression of FOXG1 contributes to TGF-beta resistance through inhibition of p21WAF1/CIP1 expression in ovarian cancer. Br J Cancer, 2009, 101: 1433–1443.
7. Ma X, Li J, Tan B. Expression of HIF-1α in hepatocellular carcinoma and its relationship with vasculogenic mimicry and clinical pathology. Chinese-German J Clin Oncol, 2013, 12: 528–531.
8. Li L, Li C, Xu X, *et al.* The effects of acetaminophen combined with radiation on the radiosensitivity of irradiated human glioma cell progeny. Chinese-German J Clin Oncol, 2009, 8: 203–206.
9. Rong L, Dai R, Cai J, *et al.* Effect of insulin-like growth factor-1 on pituitary tumor transforming gene in glioma C6 cells. Chinese-German J Clin Oncol, 2008, 7: 519–522.
10. Adesina A M, Nguyen Y, Guanaratne P, *et al.* FOXG1 is overexpressed in hepatoblastoma. Hum Pathol, 2007, 38: 400–409.
11. Martynoga B, Morrison H, Price DJ, *et al.* Foxg1 is required for specification of ventral telencephalon and region-specific regulation of dorsal telencephalic precursor proliferation and apoptosis. Dev Biol, 2005, 283: 113–127.
12. Hanashima C, Li SC, Shen L, *et al*. Foxg1 suppresses early cortical cell fate. Science, 2004, 303: 56–59.
13. Seoane J, Le HV, Shen L, *et al.* Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. Cell, 2004, 117: 211–223.
14. Seoane J, Le HV, Shen L, *et al.* Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. Cell, 2004, 117: 211–223.
15. Ahlgren S, Vogt P, Bronner-Fraser M. Excess FoxG1 causes overgrowth of the neural tube. J Neurobiol, 2003, 57: 337v349.
16. Duebgen M, Martinez-Quintanilla J, Tamura K, *et al*. Stem cells loaded with multimechanistic oncolytic herpes simplex virus variants for brain tumor therapy. J Natl Cancer Inst, 2014,106: u90.

DOI 10.1007/s10330-0314-0013-9
Cite this article as: Shao ZW, Cong BB, Sui AH, *et al*. Expression of FOXG1 is associated with the malignancy of human glioma. Chinese-German J Clin Oncol, 2014, 13: 594–599.