Immunohistochemistry analysis of Pygo2 expression in central nervous system tumors

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Abstract

Pygo2 as a Wnt signaling pathway component has been detected in multiple cancer types. In this study, we identified Pygo2 expression features by immunohistochemistry in 73 central nervous system tumor specimens, in comparison with 14 normal brain tissues and surrounding non-tumorous tissues of tumor. Our study indicated that 59% of the patient tumor specimens exhibited positive Pygo2-staining and increases intensity with the grade of malignancy, especially for WHO grade III and IV gliomas, was observed high level expression, compared with normal brain tissues. Five out of nine WHO grade III anaplastic astrocytomas and seven out of nine WHO grade IV glioblastomas showed Pygo2-positive staining. The analysis of Pygo2 gene expression by quantitative real-time PCR of additional ten fresh patient samples yielded similar results. Further studies performed with stable cell lines in vitro demonstrated that Pygo2 render cells higher proliferation rate, migration and anchorage-independent colony-forming ability in soft agar. Taken together, our studies suggest an important role of Pygo2 in brain tumor progression.

Keywords Pygo2 · Gliomas · Immunohistochemistry · Glioblastomas

Abbreviations

TCF	T cell factor
LEF	Lymphoid enhancer binding factor
PBS	Phosphate-buffered saline
PHD finger	Plant homeodomain finger

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Introduction

Pygo2, one of mammalian homologs of *Drosophila Pygopus*, is essential for early embryonic development. As an important co-activator of the Wnt/ β -catenin transcriptional complex, *Pygo2* is involved in multiple tissue morphogenesis and development, including the brain, eyes, hair follicles, lung, kidney, and pancreatic (Lake and Kao 2003; Schwab et al. 2007; Sun et al. 2014). In *Pygo2*-deficient mice, all the *mPygo2*^{-/-} animals exhibited perinatal lethality. Fourteen percent of homozygous *Pygo2*^{-/-} mice displayed cerebral abnormalities in E12.5. The role of *Pygo2* regulating normal nervous system by modulating development has been extensively studied, but possible functions during nervous system tumorigenesis remained elusive.

The mammalian tumorigenesis is normally associated with aberrant activation of signaling pathways that are generally required only during embryonic development, including Wnt and Hedgehog (Hh) signaling pathway. Ectopic expression of the genes in these pathways efficiently misregulated cell growth, proliferation, differentiation, apoptosis, invasion and angiogenesis (Taipale and Beachy 2001). As a Wnt signaling pathway component, aberrant expression of *Pygo2* has been detected in breast (Andrews et al. 2007), ovarian (Popadiuk et

al. 2006), lung (Liu et al. 2013b), esophageal (Moghbeli et al. 2013), prostate cancers (Kao et al. 2018) and gliomas (Wang et al. 2010).

As the most common primary central nervous system tumors, glioma has substantial morbidity and mortality (Behin et al. 2003; Buckner et al. 2007). Gliomas are divided into astrocytomas, oligodendrogliomas and mixed oligoastrocytomas, according to the World Health Organization (WHO) classification. High-grade astrocytomas, including anaplastic astrocytoma and glioblastoma are lethal with the median survival of a few of years and 12–15 months respectively after surgical resection and radiochemotherapy, which can be graded by their four vital histological features: increased cellularity, mitoses, endothelial proliferation, and necrosis (Dell'Albani 2008; Louis et al. 2001). Pilocytic astrocytoma and diffuse astrocytoma are typical types of low-grade astrocytomas (Buckner et al. 2007). Pygo2 expression has been reported in glioblastoma cell line and grade II and IV gliomas (Wang et al. 2010). Regarding limited data available, we investigated Pygo2 express status in various nervous system tumors with WHO grades by immunohistochemistry (IHC), real-time PCR and multiple correlation analysis in this study.

Materials and methods

Tumor samples and patient data

Brain tumor specimens for the present study were obtained from Zhongnan Hospital of Wuhan University. Utilization of human specimens was in accordance with the University's ethics commission. Histological diagnosis and grading of each tumor sample were performed according to the WHO classification by experienced neuropathologists (Table 1). Tumoral and non-tumoral lesions of the cerebrum (e.g. tissue adjacent to brain tumors) were obtained by surgical resection. Normal brain tissues were collected during surgery from head trauma patients. A total of 87 of the samples were profiled by IHC. Ten additional samples were profiled by quantitative real-time PCR. Histological classification of the specimens in this study was summarized in Tables 1 and 2.

Immunohistochemistry

IHC was performed on formalin-fixed, paraffin-embedded tissue sections using a rabbit anti-human Pygo2 polyclonal antiserum (with a dilution of 1:50, generated in our laboratory). The antiserum was raised against the amino-acid residues 88–285 of the subunit and specifically recognized the 42 kDa human Pygo2. Pygo2 antiserum was proved by immunoblot and immunofluorescence (see [Supplementary methods](#) and figure S1). In addition, the sections were finally

counterstained with hematoxylin. Negative control slides were processed in parallel with each batch of staining. Tumor sections were grouped according to semi-quantitative scoring method: The multiple score was calculated by multiplying the percentage of cells positive number ($0 < 5\%$, 1 = 5–30%; 2 = 31–55%; 3 = 55–80%; 4 = 81–100%) and stain intensity number (0: no stain, 1: slight stain; 2: moderate stain; 3: strong stain). Each sample was then classified by IHC score that graded on a scale of -, 1+, 2+, 3+: -, negative (multiple score 0); +, weakly positive (multiple score 1–3); 2+, positive (multiple score 4–6); 3+, strongly positive (multiple score 8–12). Multivariate analyses were performed subsequently.

Stable cell line

HeLa cells were cultured in DMEM medium. NIH3T3 cells were cultured in RPMI1640 medium. All cell-cultured medium were supplemented with 10% of fetal bovine serum (FBS), penicillin (100 $\mu\text{g/mL}$), and streptomycin (100 $\mu\text{g/mL}$). The HeLa and NIH3T3 cell lines that stable expressing HA-hPygo2 or empty vector were established in the previous study (De et al. 2009). The populations of HeLa and NIH3T3 cells were obtained by growing the transfectants with G418 (Invitrogen, Carlsbad, CA, USA; 400 $\mu\text{g/mL}$ and 200 $\mu\text{g/mL}$, respectively).

RNA extraction and gene expression analysis by quantitative real time PCR

Total RNA was extracted by Trizol™ reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Total RNA as templates, reverse transcriptase (Promega Inc., Madison, WI), and 500 ng of poly(T) primer were used for the synthesis of cDNA. The reaction mixture was incubated at 37 °C for 60 min. Real-time PCR was performed in a Rotor Gene 3000 (Corbett Research, Sydney, Australia) by using the DNA binding dye SYBR green (Real-time PCR Master Mix, Toyobo Co., Ltd., Osaka, Japan). A negative control lacking cDNA template was applied. For Pygo2 detection, 300 nM of forward and reverse primers was used. The thermocycling was started at 95 °C, 72 °C for 45 s with fluorescence detection; melting curve analysis was performed between 50 and 100 °C with 0.5 °C intervals. Only experiments with $R^2 > 0.990$ and a PCR efficiency between 90 and 110% were taken for analysis. The primers for real-time PCR were as follows: human β -actin forward primer, GGC ATC CAC GAA ACT AC and reverse, TAA CGG CTG TCC TAC G; human Pygo2 forward primer, CAC TCC AGA TGC CAA CA, and reverse, ACA GCC TCA CTC CAC TT (synthesized by Sangon, Shanghai, China). The relative expression of Pygo2 was calculated and normalized using the $2^{-\Delta\Delta C_t}$ method relative to β -actin. Triplicate samples were measured and arithmetic means calculated.

Table 1 Summary of tissue specimens

Diagnosis	Grade WHO	Pygo - positive	Male/ Female	Meanage(years)	NO.
Meningioma	I	13/24	6/18	51	24
Diffuse astrocytoma	II	1/9	4/5	39	9
Anaplastic astrocytoma	III	5/9	5/4	45	9
Glioblastoma	IV	7/9	6/3	51	9
Oligodendroglioma	II	4/5	2/3	32	5
Neurilemoma	III	7/10	3/1	50	4
Craniopharyngioma	I	3/3	2/1	22	3
Oligoastrocytoma	III	1/2	0/2	45	2
Pituitary carcinoma	III	0/2	1/1	47	2
Ependymal	III	0/2	1/1	19	2
Metastatic	I	1/2	1/1	58	2
Pilocytic astrocytoma	I	1/1	0/1	50	1
Papillary	I	0/1	1/0	43	1
		43/73			43
Normal brain tissue	N/A	2/6	4/2	45	6
Tumor surrounding normal tissue	N/A	2/8	3/5	33	8
		4/14			87
Total		47/87			87

Soft agar assay

5×10^4 stably transfected NIH3T3/Pygo2 or NIH3T3/vector cells were suspended in 0.5% agarose then placed on top of 0.7% agarose (Difco Laboratories, Detroit, MI) in 12 well plates. Cells were fed for 2–3 times per week with culture medium. Colonies were counted with ImageJ software (US National Institutes of Health, Bethesda, MD, USA) two or three weeks later.

Cell viability assay

NIH3T3/Pygo2 or NIH3T3/vector cells were plated in a 6 well plate (2.5×10^3 cells/each well) and incubated in DMEM with 10% FBS. After the indicated times, viable cells were collected and counted using trypan blue exclusion.

Migration assay

Cell migration assays were performed using transwell chambers (BD Biosciences, Bedford, MA, USA). HeLa/Pygo2 or HeLa/vector cells (1×10^5) in 200 μ L of serum-free DMEM were seeded in the upper chamber and 800 μ L of medium supplemented with 10% of FBS was added to the lower chamber. The migrated cells were fixed and stained with 0.1% of (*w/v*) crystal violet 24 h later. Five randomly selected fields were photographed and the numbers were counted.

Cell apoptosis assay

Apoptosis of stable cells was assessed by examining nuclear morphology in Hoechst 33,342 stained cells. HeLa/Pygo2 or HeLa/vector cells were treated with microtubule destabilizing agent vinblastine (2 mg/mL) for 48 h. Then, cells were stained with 1 μ g/mL of Hoechst 33,342 (Sigma Chemical Co, Louis, MO, USA) and the fraction of cells exhibiting an apoptotic nuclear morphology characterized by chromatin condensation and/or apoptotic bodies. Images were captured with a fluorescence microscope (Leica DMIRB; Leica Microsystem, Wetzlar, Germany). Vinblastine was purchased from Sigma (Sigma Chemical Co, Louis, MO, USA).

Plasmids and transcriptional activity assay

The truncated hPygo287–406, Pygo1–328, Pygo240–406, Pygo328–406, Pygo87–328, full-length hPygo2, and site mutations of full-length hPygo2 were subcloned into pBIND vector from the CheckMate mammalian two-hybrid kit (Promega, Madison, WI, USA). Site-directed point mutations in the Pygo2 PHD domain were engineered by following the standard overlap extension PCR-mediated mutagenesis procedures. The fusion protein contained the following mutations: T362A; A365V; L369A; W376A; W353A; F354A; L345A; V337A; C330A; C381A; C351A; H356A; H356C; C359A and C359H. Appropriate reading frame fusions for all constructs created by PCR were confirmed with DNA sequencing.

Table 2 Summary of IHC score of gliomas and meningioma

Diagnosis	Pygo2 positive staining	Pygo2 staining in tensity			
	Percentage	-	+	++	+++
Normal					
Normal brain tissue	2/6	4	2	0	0
Tumour surrounding normal tissue	2/8	6	2	0	0
Total	4/14	10	4	0	0
Meningioma					
Meningioma (I)	13/24	11	12	1	0
Glioma					
Diffuse astrocytoma(II)	1/9	8	1	0	0
Anaplastic astrocytoma(III)	5/9	4	4	1	0
Glioblastoma(IV)	7/9	2	3	3	1
Oligodendroglioma (II)	4/5	1	4	0	0
Oligoastrocytoma (III)	1/2	1	1	0	0
Pilocytic astrocytoma(I)	1/1	0	1	0	0
Ependymal (III)	0/2	2	0	0	0
Total	19/37	18	14	4	1
Total	36/75	39	30	5	1

293 cells were plated in a 24 well plate and transfected with pBIND-Pygo2 and pG5luc plasmids. Renilla-TK (Promega, Madison, WI) was co-transfected in each case for internal control. Luciferase assay was performed by the dual luciferase assay kit according to the manufacturer's instructions (Promega, Madison, WI, USA). The results were expressed as average relative firefly luciferase activity, normalized to renilla luciferase activity.

Statistical analysis

Statistical comparisons were performed using GraphPad Prism version 5.0 for Windows software (GraphPad Software, San Diego, CA, USA). Data are presented as means $\pm$ SEM. Statistical analysis was performed by using ANOVA or two-tailed Student's t-test when appropriate. mRNA expression levels were compared using Mann–Whitney independent t-test. Comparison of groups was performed by Pearson chi-square test (Fisher's exact test was applied if the number was fewer than five). The two-way ANOVA was used to compare the effect of Pygo2 in cell viability assay. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. $P < 0.05$ was considered statistically significant.

Results

IHC analysis for Pygo2 in human central nervous system tumors

We performed IHC on paraffin-embedded specimens from 73 patients to determine the expression level of Pygo2. 14 normal

brain and surrounding non-tumorous tissues were included as controls. In our study, total 43 out of 73 tumor specimens stained positive for Pygo2 (59%), which is significantly higher than that observed in control specimens, four positive out of 14 (29%, Table 1).

For all grades of gliomas, IHC determined Pygo2 expressed in 19 out of 37 specimens (51.4%, Table 2). A number of malignant glioblastomas (WHO grade IV) showed strong nuclear staining and weak cytoplasmic staining, compared with normal brains (Fig. 1a, b and d). Furthermore, Pygo2 positive staining also observed in anaplastic astrocytomas (WHO grade III) and oligodendrogliomas (WHO grade II, Fig. 1c and e). As shown in Table 2, IHC staining intensity and frequency of Pygo2-positive cell was indicated by IHC scores (see Methods section). Our study demonstrated that in low-grade astrocytomas, including diffuse astrocytoma (WHO grade II) and pilocytic astrocytoma (WHO grade I), total two out of ten WHO grade I and II (20.0%) showed weak expression of Pygo2, while high-grade astrocytomas, five out of nine WHO grade III astrocytomas (55.6%) and seven out of nine WHO grade IV glioblastomas (77.8%) expressed Pygo2 (Table 2), indicating that more cells expressed Pygo2 in malignant astrocytomas than in low-grade astrocytomas. Almost all strong staining (2+ and 3+) of Pygo2 was present in glioblastomas. In addition, 4/5 of oligodendroglioma (WHO grade II) express Pygo2.

As shown in Fig. 2a, the distribution of Pygo2 IHC scores for WHO grade I-II (mean, 0.50) and WHO grade III (mean, 0.53) populations were similar. WHO grade I-II and III groups were reflected higher median scores when they are compared with normal brain group (mean, 0.29), but they had no significant difference. However, a marked difference was observed

Fig. 1 IHC detection of Pygo2 in human gliomas and normal brain specimens. **a** Glioblastoma (WHO grade IV) showed strong (+++) Pygo2-positive staining. **b** Glioblastoma and **(c)** Anaplastic astrocytoma (WHO grade III) showed moderate (++) Pygo2 staining. **d** Glioblastoma and **(e)** Oligodendroglioma (WHO grade II), Pygo2 staining was weak (+). **f** Surrounding non-tumorous tissues is negative (–) for Pygo2 staining. Bars 50 μ m

when comparing the distribution of IHC scores between WHO grade IV (mean, 1.33) and control group ($P < 0.01$).

Statistical comparison among patients with different grades, histological subtypes, and Pygo2-enriched characteristics were listed in Table 3. The data showed significant differences of

Pygo2 expression between glioma histological subtypes based on correlated multiple testing ($P = 0.0059$). There were no distinct differences between gliomas and normal brain ($P = 0.1037$). In the cases of WHO grade IV glioblastomas, Pygo2 expression pattern significantly differed from normal brain ($P = 0.0094$), although that no difference observed from other glioma subtypes.

Meningioma is a typical tumor from arachnoid cap cells accounting for one-third of central nervous system tumors (Ohgaki and Kleihues 2005). The majority (90%) of meningiomas is benign meningioma (WHO Grade I) (Fathi and Roelcke 2013; Wiemels et al. 2010). Here, we included 24 benign meningiomas specimens in our study, 13 meningiomas (WHO grade I, 54.2%) showed Pygo2-positive staining (Table 1). Although Pygo2 expression in meningiomas was higher than in normal brain, the difference did not reach the statistical significance for correlated multiple testing ($P = 0.1812$, Table 3).

Detection of Pygo2 expression with real-time PCR

To validate our findings, we profiled ten additional samples with real-time PCR, including seven astrocytoma tumors, and three normal brain controls. Astrocytoma tumors displayed the greatest disparity on Pygo2 mRNA level, and significantly higher than normal brain. This observation was consistent with the IHC data that gliomas expressed high level of Pygo2 (Fig. 2b).

Cell viability, migration and colony formation assay

To further address whether Pygo2 has oncogenic potential, we have generated Pygo2 stable expression cell lines both in non-malignant (NIH3T3) and malignant (HeLa) cells, respectively.

Fig. 2 Pygo2 expression pattern in gliomas. **a** Statistical analysis of Pygo2 IHC score in normal brain ($n = 14$), Grade I–II ($n = 15$), Grade III ($n = 13$), and Grade IV ($n = 9$) gliomas, P -values by two-tailed t -test. **b** Quantitative analysis of Pygo2 mRNA expression in astrocytoma ($n = 7$)

compared with normal brain tissues ($n = 3$, P -value was calculated by Mann–Whitney independent t -test). Data shown were means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$; ns, no significance

Table 3 Comparison of Pygo2 expression in different WHO grade of tumor

Characteristic	Total	Pygo2 positive staining		P value (χ^2 test)
		-	+	
Total				
Tumor surrounding/normal tissue	14	10	4	0.1037
Gliomas	37	17	20	
Histological subtype				
Glioblastoma(IV)	9	1	8	0.0059
Anaplastic astrocytoma(III)	9	4	5	
Diffuse astrocytoma(II)	9	8	1	
Oligodendroglioma (II)	5	1	4	
Glioma grade(WHO)				
Tumor surrounding/normal tissue	14	10	4	0.0360
Grade I-II	15	9	6	
Grade III	13	7	6	
Grade IV	9	1	8	
Comparison two groups				
Tumor surrounding/normal tissue	14	10	4	0.1812 (§)
Meningioma (I)	24	11	13	
Tumor surrounding/normal tissue	14	10	4	0.0094 (§)
Glioblastoma(IV)	9	1	8	
Tumor surrounding normal tissue	14	10	4	0.3826 (§)
Anaplastic astrocytoma(III)	9	4	5	
Tumor surrounding/normal tissue	14	10	4	0.6106 (§)
Diffuse astrocytoma(II)	9	8	1	
Tumor surrounding/normal tissue	14	10	4	0.1108 (§)
Oligodendroglioma (II)	5	1	4	

§Fisher's exact test

Colonies formation assay is characterized as an indicator of the transformed phenotype. We compared the colony formation ability of Pygo2 stable expression mouse fibroblast NIH3T3 cells with control cells. Many colonies were developed from NIH3T3/Pygo2 cells in soft agar medium, but almost no visible colony was observed in NIH3T3/vector cells (Fig. 3a, b). This observation indicated that Pygo2 promote cell anchorage-independent growth.

As shown in Fig. 3d, faster proliferation rate was observed in NIH3T3/Pygo2 cells in cell viability assay. We further conducted the cell death assay. The cell death induced by anti-tumor chemical drug vinblastine was determined by Hoechst 33,342 staining (Fig. 3c). Typical apoptotic morphology such as condensed or fragmented nuclei was observed in HeLa/vector cells, but less in HeLa/Pygo2 cells. This result was consistent with our previous study that HeLa/Pygo2 cells were capable of overcoming cell apoptosis caused by vinblastine (De et al. 2009).

In migration assay, the number of migrated cells in the Pygo2-overexpressing group was clearly increased compared with the number in the control cell (Fig. 3e, f). Overall, Pygo2 exhibited its oncogenic potential through promote cell

oncogenic transformation, proliferation, migration, and anti-apoptotic effect.

Transcriptional activation of Pygo2

Pygo has two conserved domains, an N-terminal homology domain (NHD) and a C-terminal PHD zinc finger motif. To test whether these motifs are essential for the transcriptional function, we generated a set of truncated and mutated forms of Pygo2 for transcriptional activity testing. First, five deletions were conducted to determine the minimal transcriptional activity domains. Pygo87–406 is harboring NHD domain deletion of Pygo2, and Pygo1–328 bear a deletion of PHD domain. Pygo240–406 and Pygo328–406 were truncated the Pygo2 protein just after N-terminal 240- and 328-amino acid, respectively. Pygo87–328 lacked of both NHD and PHD domain. The construct pBIND-Pygo contained the fusion of the GAL4 DNA-binding domain and different-length Pygo2 efficiently stimulates transcription of a luciferase reporter. We set full-length Pygo2 transcription activity as 100%, the percentage of activity of all truncated and mutated forms Pygo2 was calculated. Deletion of PHD domain (Pygo1–328) showed

Fig. 3 Cells overexpressing Pygo2 acquired characteristics of malignancy. **a, b** Colony-formation assay in NIH3T3 cells stably transfected with Pygo2, or vector alone as described in *Materials and Methods*. Results were means of triplicates $\pm$ SEM from three independent experiments, *P*-value by two-tailed *t*-test. Bar 100 μ m. **c** Vinblastine induced apoptosis in stable cells. Representative images of Hoechst 33,342 staining were visualized at 48 h after vinblastine treatment. HeLa/vector cells exhibit apoptotic nuclear morphology. Bar 30 μ m. **d** Cell viability of NIH3T3/Pygo2 (circles) or NIH3T3/vector

(squares) stable cells was determined at the time points shown by trypan blue dye exclusion. The data presented were the means of triplicate samples $\pm$ SEM. Representative data from one of three independent experiments are shown. *P*-value by two-way ANOVA. **e** Migrating cells were stained with 0.1% crystal violet and visualized by microscopy. Representative images of migrated cells and statistical analysis of cell migration (**f**) of HeLa stable cell lines. Results were means of triplicates $\pm$ SEM from two independent experiments, *P*-value by two-tailed *t*-test. Bar 50 μ m. ***P* < 0.01, ****P* < 0.001

80% reduced transcriptional activity. Deletion of NHD domain (Pygo87–406) reduced 50% transcriptional activity. The reductions of transcriptional activity caused by long-range deletion (Pygo240–406, remain 33% and Pygo328–406, remain 25%) were more pronounced than that of Pygo87–406 (Fig. 4b).

To further investigate the role of PHD finger motif in Pygo2 transcriptional function, we expressed Pygo2 protein containing three different type site mutations (Fig. 4a). In the first type, the five zinc-coordinating residues in PDH domain typical C4HC3 (four cysteines, one histidine, and three cysteines) structure were mutated to Alanines (C330A, C381A, C351A, H356A, and C359A), resulted in a one-fold of decrease in transcriptional activity, compared to the full-length Pygo2 construct. It suggested that correct coordinate zinc ions for stabilizing the PHD finger structure were important for

transcriptional activity of Pygo2. The mutation H356C and C359H substitute the Histidine/Cysteine with another zinc-coordinating residue displayed 60% activity reduction. Based on the structure, the second type of mutated residues (T362A, A365V, L369A) were the key sites influence Pygo2 binding to Legless/Bcl-9 (which in turn binds to the β -catenin) (Kramps et al. 2002), these mutations also caused 50% activity reduction, this could be because of Pygo2 exert transcriptional activity through the interaction with β -catenin complex. Finally, mutations (W376A, W353A, F354A, and V337A) were expected to disrupt the hydrophobic core of the PHD finger, resulting in varying activity reduction (20–50%). The mutation of L345, a conserved hydrophobic residue in the neighborhood of C3, showed no significant change in activity suggesting that L345 did not participate in hydrophobic core of PHD domain (Fig. 4b).

Fig. 4 Identification of domains and residues are critical for Pygo2 transcriptional activities. **a** Sequence alignments of the PHD domain of *Drosophila* and human Pygo. Blue color: C4HC3 bone. Red color: hydrophobic amino acid. **b** The diagram shows transcriptional activities of full-length, mutated and truncated human Pygo2 proteins that examined in this work. After transfection 24 h, cells were lysed, and protein lysates were assayed for luciferase activities. Bars indicate mean $\pm$ SEM of triplicates in one of two represent experiment

Discussion

In this study, we have found that Pygo2 expression in malignant gliomas, especially in glioblastoma, was significant higher than the normal brain. Strong Pygo2 immunoreactivity was present in nuclei of tumor cells in most glioma histologic subtypes. Gliomas are the most common types of primary human brain tumors that start from glial cells (Louis et al. 2001). Astrocytomas, oligodendrogliomas and ependymomas are the common gliomas (Buckner et al. 2007). The

correlation analysis between Pygo2 expression status and different variables indicated that staining pattern could help discriminate glioblastoma from other subtypes. Recently, lots of effort to identify prognostic factors for glioblastoma and other glioma subtypes has focused on genetic factors and molecular markers. According to our data, we propose that Pygo2 as a potential biomarker that can be used to classify gliomas malignancies and to define clinical glioblastoma characteristics.

In addition, our data showed that Pygo2 protein aberrations seemed to not associate with benign meningioma. This

observation was different with that gliomas expressed high level of Pygo2. Unlike gliomas, etiological study of meningioma clearly indicated that the risk factors including ionizing radiation, hormones and head trauma closely associated with meningioma incidence (Fisher et al. 2007; Longstreth et al. 1993; Wiemels et al. 2010).

Previous research has demonstrated that the deletion of Pygo2 delayed tumor onset in Wnt-1 transgenic mice that spontaneously developed extensive mammary adenocarcinomas (Watanabe et al. 2014). Suppression of Pygo2 expression inhibited ovarian, lung cancer cell and glioma cells proliferation (Liu et al. 2013b; Wang et al. 2016). The Pygo2 oncogenic potential was also evaluated by xenograft assays in immunodeficient mice both for wild and knockdown Pygo2 epithelial ovarian cancer cells. Pygo2 knockdown cells formed smaller tumors than control cells (Popadiuk et al. 2006). The role of Pygo2 in carcinogenesis was further investigated in this study. Conclusions are as follows: 1) Stable expression of Pygo2 promoted cell proliferation and migration. 2) Pygo2 promoted non-malignant cell anchorage-independent growth ability. 3) HeLa cells with overexpression of Pygo2 acquired resistance to anti-tumor agent vinblastine. Given Pygo2 exerts regulation in the developing brain, an aberrant expression of Wnt signal component Pygo2 in gliomas may reveal a link between brain developmental processes and tumorigenesis. Plenty of small molecules or Wnt pathway-targeted antibodies showed significant antitumor activity by attenuate Wnt signaling in various preclinical experiments, and entered clinical trials (Blagodatski et al. 2014; Lee et al. 2016; Liu et al. 2013a; McCord et al. 2017). Because of the poor prognosis of patients with glioblastomas, the research of new treatment strategy becomes very important. Our research provided a possibility that disrupting Pygo2 could serve as a novel strategy for the design of anti-gliomas agent.

Pygo2 as a DNA-binding protein exerts transcriptional co-activator function in Wnt-dependent and -independent pathway (Jessen et al. 2008). Upon Wnt signaling stimulation, the β -catenin degradation is blocked by Dishevelled (Dsh), and then β -catenin accumulates and translocates into the nucleus, where it binds Pygopus via Legless/Bcl-9. Finally, the complex bound to members of the TCF/LEF family, triggers the transcriptional activation of Wnt target genes (Cadigan 2002). Pygo functions as a transcriptional co-activator, the autonomous activation of Pygo roots in its two domains (NHD domain and PHD domain). We monitored the effect of different domains and residues on transcriptional activity. Mutations of PHD zinc-coordinating residues alter the metal ion position caused the dramatic reduction of transcriptional activity. Previous study reported that point mutations of conserve residues of T782A, A784V, and L789A (corresponding to T362A, A365V, L369A in human Pygo2 sequence) disrupt the binding between mPygo2 and Legless (Townsend et al. 2004). Our data showed that T362A, A365V, L369A

mutations drastically reduces the transcriptional activity, suggesting that Pygo exert transcriptional function required the recruitment of Legless/Bcl-9 or TCF complex. Stadeli and Basler described the conserved amino acids N, P, and F in NHD domain had the crucial effect on Pygo transcriptional activation capacity (Stadeli and Basler 2005). Crystallographic analysis showed that site L380 (corresponding to L369 in human) of mouse Pygo1 is essential for Pygo dimerization (Nakamura et al. 2007). Our data has shown that 50% reduction of transcriptional activity in L369A mutation. Taken together, these results strongly support the notion that the region between amino acids 328 and 406 forms a PHD type zinc finger which is critical for Pygo2 transcriptional activity.

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