

Original Article

Amplification of PINX1 in glioblastoma promotes cell proliferation and is targeted by miR-627

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Abstract: Objective: PIN2/TRF1-interacting telomerase inhibitor 1 (PINX1) is a novel cloned gene that plays a vital role in maintaining telomeres length and chromosome stability. In this study, we investigated the expression, function and mechanisms that governing its aberrant expression in human glioblastoma cells. Methods: A total of 46 glioblastoma tissues and 10 non-cancerous brain tissues were enrolled in this study. The expression pattern of PINX1 was detected by quantitative real-time PCR and immunohistochemistry. By conducting siRNA mediated PINX1 inhibition and plasmid overexpression, cell proliferation was evaluated by Edu staining and MTT assay in U87 and SNB19 cells. Moreover, we studied the novel relationship between PINX1 and miR-627 in glioblastoma cells. Results: Compared with non-cancerous normal brain tissues, expression of PINX1 mRNA and protein levels were remarkably amplified in glioblastoma. Cellular experiments demonstrated that PINX1 promoted cell proliferation. Further studies suggested miR-627 was the direct upstream regulator that post-transcriptionally targeting PINX1. The expression of PINX1 and miR-627 negatively correlated with each other, and miR-627 exhibited decreased status in glioblastoma tissues. Conclusion: Amplified PINX1 serves as a potent oncogene by promoting cell proliferation in glioblastoma, and miR-627 directly targets PINX1, thus revealing a novel therapeutic strategy for glioblastoma patients.

Keywords: PINX1, amplification, glioblastoma, cell proliferation, miR-627

Introduction

PIN2/TRF1-interacting telomerase inhibitor 1 (PINX1) is known as a potent telomerase inhibitor that was firstly identified in 2001 [1]. Unlike other telomere-associated proteins, PINX1 is unique for the reason that it directly interacts with the telomerase catalytic component TERT and suppresses the enzymatic activity of telomerase [1]. Accumulating reports have considered PINX1 as a tumor suppressor because of its conserved effects on inhibiting telomerase activation and telomeres elongation in cancer cells [2-5]. Evidences from other epithelial malignancies, including gastric cancer [5, 6], prostate cancer [7] and ovarian cancer [8], have shown that PINX1 expression is down-regulated and associated with poor prognosis. Moreover, PINX1 overexpression was verified to be able to significantly suppress cell proliferation, or induce apoptosis in some types of cancers [9, 10]. However, there are still some research-

es showing the opposite molecular status of PINX1 gene and its expression patterns in other types of tumors, like in esophageal squamous cell carcinoma (ESCC) and cervical squamous cell carcinomas (CSCC) tissues [11, 12]. These indicate that the variations of expression pattern and/or functions of PINX1 in tumorigenesis and progression are complicated, and may be tumor-type specific.

Glioma accounts for more than 70% of all primary neoplasms that developed in the central nervous system [13]. Glioblastoma, also abbreviated as GBM, is the grade IV of all gliomas according to the WHO classifications. It represents the most aggressive and deadly primary brain tumor on account of its fast growth and frequently spreading to nearby brain tissue [14, 15]. Surgical resection, followed by radiotherapy and chemotherapy remains as the common therapy option. Despite the progress in understanding the features of this disease, the prog-

nosis actuality remains unsatisfying. The median life expectancy of all GBM patients after diagnosis remains less than 12 months [16].

Recently, Bai et al firstly characterized the expression and prognostic value in human glioma [17]. They found that PINX1 protein levels were remarkably upregulated in gliomas, and PINX1 expression may serve as a prognostic and predictive biomarker for gliomas [17]. Tracing along this finding, we set out to investigate the function and mechanism of PINX1 in GBM.

Materials and methods

Patients and tissues

The surgical specimens of 10 normal brain tissues and 46 glioblastoma tissues were collected in Cangzhou Central Hospital from January 2015 to December 2016 with written consent. For total RNA extraction, tissues were frozen in liquid nitrogen immediately followed by stored in -80°C.

Immunohistochemistry

For immunohistochemistry, tissues were formalin-fixed and processed by paraffin-embedded before used. The IHC staining was performed on the sections of (5 µm thickness) according to the standard ABC protocol with the PINX1 antibody (Santa Cruz).

Cell culture

Normal human astrocytes and human glioblastoma cell lines U87, U343, U251 and SNB19 were obtained from laboratory preservation. All cell lines were cultured in DMEM medium (Hyclone) supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin-streptomycin (SolarBio). All the cells were cultured in a humidified atmosphere at 37°C with 95% air and 5% CO₂.

RNA isolation and quantitative real-time PCR

Total RNA and microRNAs were extracted from the tissues and cells mentioned above using TRIzol reagent (Invitrogen), according to the manufacturer's protocol. First-strand cDNA was synthesized from 1 µg of total RNA using reverse transcriptase (Takara). GAPDH was used as an internal control of genes. Special primers (RiboBio) were used for the miR-627 RT reaction, and U6B was used as an internal control

of miRNA. Quantitative real-time PCR analysis of PINX1 or miR-627 expression was carried out by 2^{-ΔΔCT} method using 5 µl cDNA and the SYBR Green Master Mix (TOYOBO), according to the manufacturer's instructions.

Short interference RNA

A short interference RNAs (siRNAs) mixture targeting PINX1 (siPINX1) and control siRNA oligos (siNC) were purchased from Santa Cruz. Transient transfections was performed using Lipofectamine2000 (Invitrogen), according to the manufacturer's instructions.

Plasmid construction

The coding sequence of PINX1 was amplified from U87 cDNA by reverse transcription PCR and inserted into pcDNA3.1-myc/His (A+) expression vector without myc-tag.

Western blot

Total proteins from all the cell samples were extracted using RIPA (Beyotime) buffer containing 1 mM PMSF (Beyotime). 30 µg of each protein was loaded on 9% SDS polyacrylamide gels for electrophoresis. Then proteins were transferred to NC membranes (Millipore). After being blocked by 5% non-fat milk, the membranes were incubated with PINX1 or GAPDH antibodies (Santa Cruz) and HRP conjugated secondary antibodies (Santa Cruz). GAPDH was used as internal controls. HRPs on the immune complex were visualized by the ECL Plus Kit (Thermo).

MTT assay

The role of PINX1 in growth rate of U87 and SNB19 cells was determined by the MTT assay. Briefly, cells were plated in 96-well plates at a density of 2000 cells per well. Cells were incubated with 0.5% MTT (Sigma). 4 h later, cells were lysed by DMSO (Beyotime) and the absorbance was determined by a microplate reader at 490 nm.

Edu staining assay

For Edu assay, 50 mM of the reagent from Cell Light Edu DNA imaging Kit (RiboBio) was added two days after transfection, and the cells were stained according to the manufacturer's protocol. The Edu-positive cells were stained by red and cells stained by Hoechst were blue.

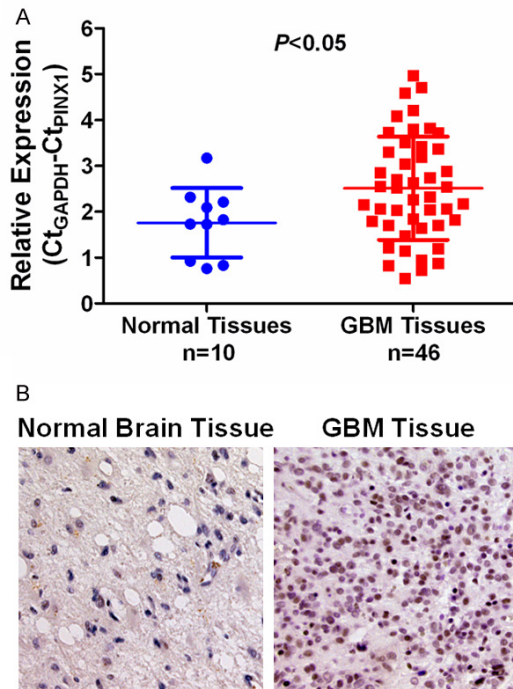


Figure 1. PINX1 was amplified in mRNA and protein level in GBM tissues. A. PINX1 was up-regulated in 46 GBM tissues compared with 10 normal brain tissues by qRT-PCR ($P<0.05$). B. Immunohistochemistry staining showed the up-regulation of PINX1 at protein level in GBM tissue section compared with normal brain tissue section ($\times 100$).

MiR-627 target prediction

Candidate targets of miR-627 were predicted by free online tools of TargetScan (<http://www.Targetscan.org/>) and miRBase (<http://www.mirbase.org/>).

Dual-luciferase reporter assays

The PINX1 3'-UTR containing the predicted binding site for miR-627 was amplified and cloned into pmir-GLO vector. PINX1 3'-UTR containing mutant binding site for miR-627 was sub-cloned by site-directed mutagenesis PCR method. 48 hours after transfection, luciferase activity was detected using a dual-luciferase reporter assay system and normalized to renilla activity.

Statistically analysis

Statistical analysis was performed using GraphPad v5. All values were expressed as mean \pm standard deviation (SD). Differences between groups were calculated using the Grouped Student's *t*-test. Correlation between gene and

microRNA expression was analyzed by linear regression analysis. A *P* value <0.05 was considered as statistically significant.

Results

Amplification of PINX1 mRNA and protein in GBM tissues

Although it was shown by another group that PINX1 was upregulated in gliomas [17], we still firstly confirmed this result in our GBM tissues. A total of 46 GBM patients and 10 non-cancerous normal brain tissues were received from the clinic. Quantitative real-time PCR analysis revealed that, PINX1 mRNA was obviously increased in the GBM tissues when compared with the normal brain tissues ($P<0.05$, **Figure 1A**). Additionally, our immunohistochemistry result showed that PINX1 was mainly located in the nucleus, almost absent in the normal brain tissue while strongly positive staining in the tested GBM tissue (**Figure 1B**). These data support that PINX1 was amplified at both mRNA and protein levels in GBM tissues.

PINX1 promotes cell proliferation in U87 and SNB19 cells

To investigate the possible biological effects of PINX1 in GBM cell proliferation, we also determined its expression level in some GBM cells. As shown in **Figure 2A**, when compared with normal human astrocytes, PINX1 was mostly abundant in the U87 cell line, while modestly expressed in the SNB19 cell line. Then we performed loss-of-function assay in U87 cells while gain-of-function in SNB19 cells. Western blot validation showed the effective knockdown or overexpression of PINX1 in the respected cells (**Figure 2B**). Then we carried out Edu and MTT assays to evaluate the cell proliferation ability influenced by PINX1. Both methods gave the same phenotype: 1) intrinsic PINX1 knockdown resulted in slowing down of cell proliferation in U87 cells, and 2) forced expressing PINX1 in SNB19 cells caused accelerated growth rate (**Figure 2C** and **2D**).

miR-627 directly targets PINX1

Given that microRNAs (miRNAs) play key roles in regulating gene expression at the post transcriptional level, we then questioned if some miRNA targeted PINX1, which might be utilized as the GBM therapy target. By using free online

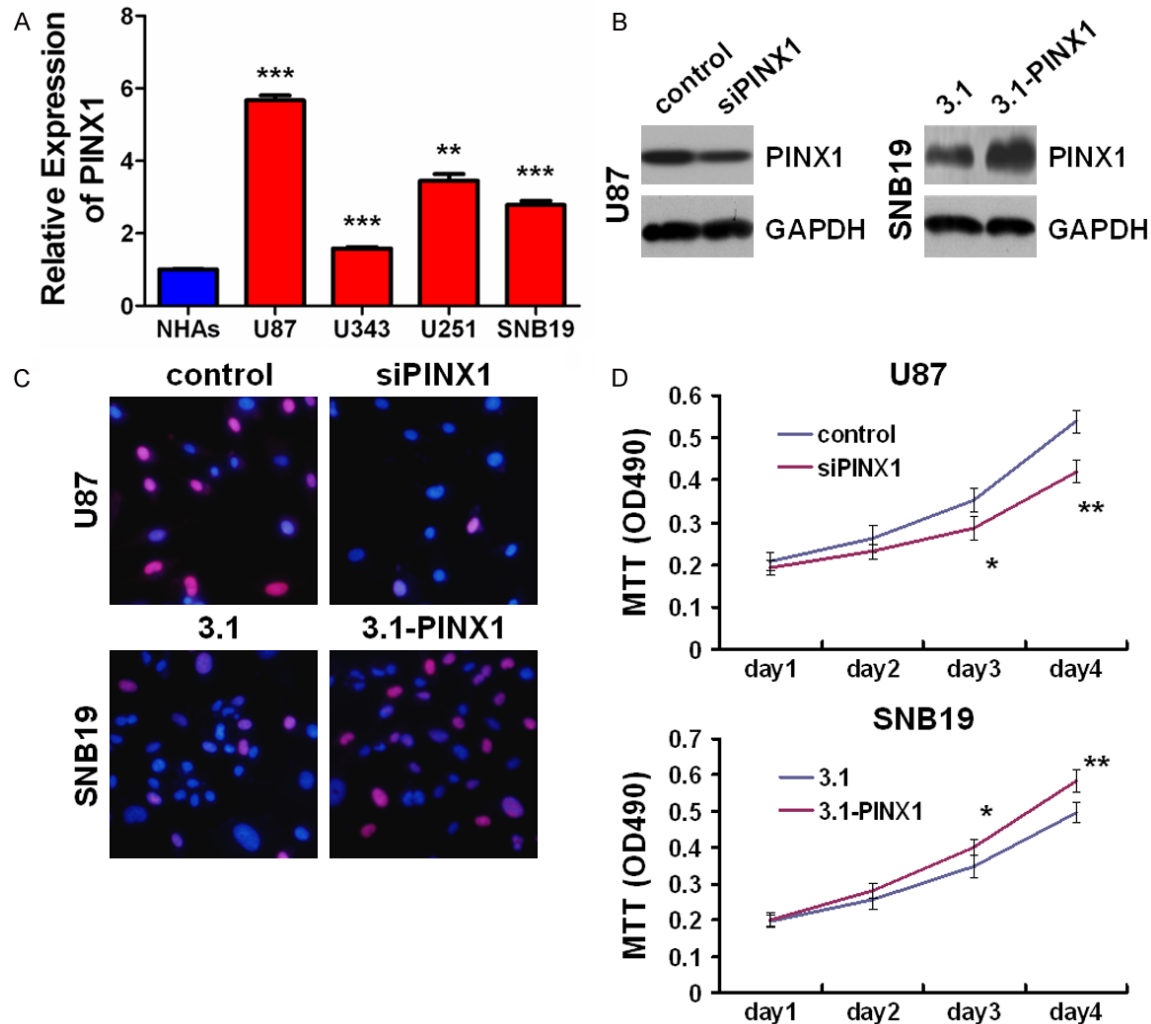


Figure 2. Upregulated PINX1 promoted cell proliferation in U87 and SNB19 cells. **A.** Fold changes ($2^{-\Delta\Delta C_t}$ values) by qRT-PCR showed increased expression of PINX1 mRNA in four GBM cell lines compared with normal human astrocytes (NHAs). Expression levels were normalized for GAPDH. **B.** Western blot indicated down-regulation of PINX1 in PINX1 siRNAs transfected U87 cells and up-regulation of PINX1 protein in the 3.1-PINX1 plasmid transfected SNB19 cells in comparison with control cells. **C.** Edu-positive representative images in the indicated cells assessed by Edu assay after transfecting siPINX1 mixture in U87 cells or 3.1-PINX1 plasmid in SNB19 cells. **D.** MTT assay showed a lower growth rate in the siPINX1 transfected U87 cells and a higher growth rate in 3.1-PINX1 transfected SNB19 cells than control cells. *: $P < 0.05$, **: $P < 0.01$.

target prediction tool TargetScan Release 7.1 [18], we found putative seed-matching sites (indicated in **Figure 3A** in blue) for miR-627 in the 3'-UTR of PINX1 (**Figure 3A**). Luciferase reporters containing wild-type and mutant sites (indicated in **Figure 3A** in red) were constructed. Then luciferase activities of wild-type and mutant PINX1 reporters were detected in U87 cells after co-transfection of miR-627 mimics. The results showed that the wild-type PINX1 reporter had significantly lower levels of luciferase activity in miR-627-overexpressing cells, while the mutant PINX1 reporter had similar le-

vels between miR-NC and miR-627-overexpressing cells (**Figure 3B**). Furthermore, we determined the protein and mRNA expression of PINX1 in miR-627-overexpressing cells, and found that PINX1 expression was significantly reduced in miR-627-overexpressing U87 and SNB19 cells (**Figure 3C** and **3D**).

Decreased expression of miR-627 indicates its tumor suppressing role in GBM

Since we proved that miR-627 was the direct regulator of PINX1 in GBM cells, we then inves-

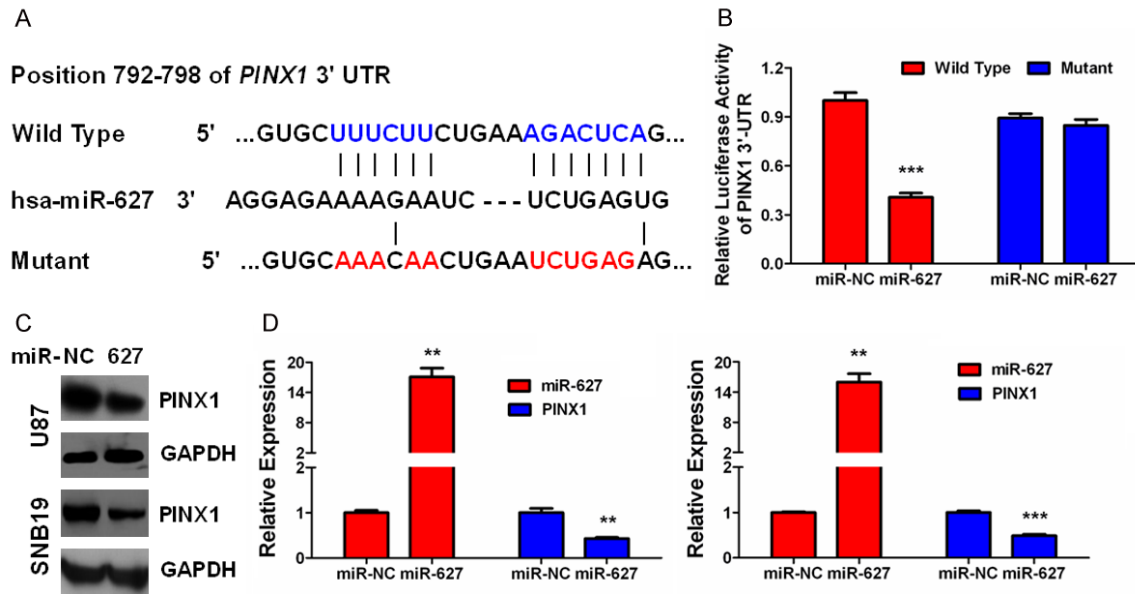


Figure 3. miR-627 directly targeted PINX1 and negatively regulated its expression. A. Predicted miR-627 binding sites in the 3'-UTR of PINX1 by miRBase or TargetScan (in blue) and the designed mutant 3'-UTR in which miR-627 binding sites were mutated (in red). B. Dual luciferase assay results showed the inhibitory effect on PINX1 3'-UTR activity while over-expressing miR-627. U87 cells were co-transfected with the recombinant pmir-GLO plasmids containing PINX1 wild type or mutant 3'-UTR and miR-627 mimics or miR-NC controls. The firefly luciferase activities were normalized by the renilla luciferase activities. ***: $P < 0.001$. C. Western blot indicated down-regulation of PINX1 in miR-627 mimics transfected U87 and SNB19 cells in comparison with miR-NC transfected control cells. D. Fold changes ($2^{\Delta\Delta Ct}$ values) by qRT-PCR showed decreased expression of PINX1 mRNA when over-expression miR-627 mimics in U87 and SNB19 cells. Expression levels were normalized for U6 or GAPDH.

targeted miR-627 expression in GBM tissues. Results showed that miR-627 expression levels in GBM specimens were much lower than those in normal brain tissues ($P < 0.01$, **Figure 4A**), indicating miR-627 acts as a tumor suppressor in GBM. Spearman's correlation analysis found the negative correlation between the expression levels of PINX1 and miR-627 in GBM tissues (**Figure 4B**). Finally, we examined the biological effects of miR-627 in GBM cells by MTT assay. The result shown in **Figure 4C** indicated significant reduced cell proliferation ability of miR-627-overexpressing cells compared with cells transfected with miR-NC.

Discussion

PINX1 has been identified as an endogenous telomerase inhibitor and a major haplo-insufficient tumor suppressor gene, since its suppressive roles had been confirmed in the heterozygous (PINX1^{+/−}) mice, which had increased opportunities for breast, lung, and gastrointestinal carcinomas development [19]. However, several studies have also showed the different

and even contradictory roles of PINX1 in telomerase activity and cancer cells [20, 21]. This reminds us that the function of PINX1 might be complicated and highly depended on the types of cancers. Nevertheless, the exact expression and function of PINX1 remain unclear.

Recently, one report characterized the expression and prognostic value of PINX1 in human gliomas [17]. They found that PINX1 protein levels were remarkably upregulated in gliomas, and PINX1 expression may serve as a prognostic and predictive biomarker for gliomas [17]. Besides, Danussi et al previously presented a new algorithm called Multi-Reg, which conducted computational integration of copy number variation, expression and mutation data from large datasets of human GBM [22]. In their study, they identified that PINX1 was among the candidate drivers located in the amplified chromosome regions [22].

Herein, on the basis of human GBM tissues and normal brain tissues from the clinic, we determined the expression level of PINX1 in these

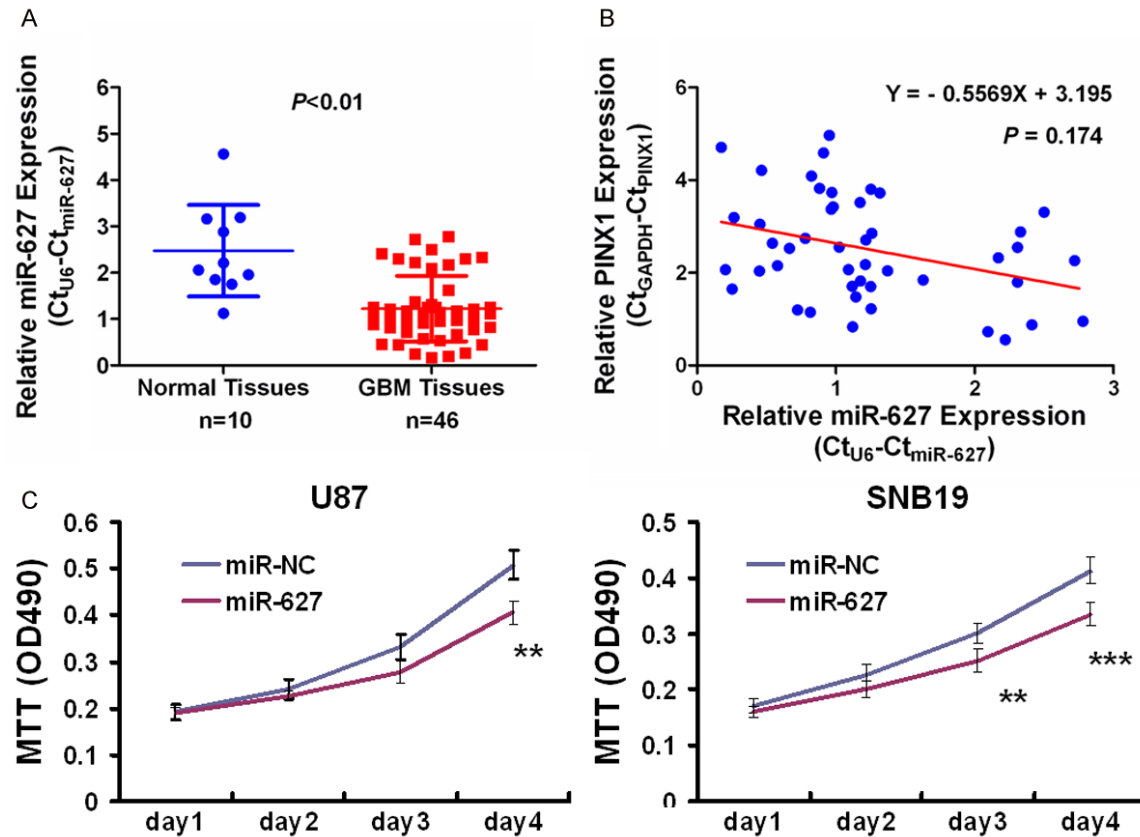


Figure 4. miR-627 was down-regulated in GBM tissues and inhibited GBM cell proliferation. A. MiR-627 was down-regulated in 46 GBM tissues compared with 10 normal brain tissues by qRT-PCR ($P < 0.01$). B. Negative correlation of miR-627 and PINX1 expression in 46 GBM tissues by linear regression analysis ($R^2 = 0.1218$). C. MTT assay showed a lower growth rate in the miR-627 mimics transfected U87 and SNB19 cells than miR-NC transfected control cells. **: $P < 0.01$, ***: $P < 0.001$.

GBM tissues, and also in GBM cell lines. We found that PINX1 mRNA and protein levels were both elevated in GBM tissues when compared with the normal brain tissues. We did not focus on its DNA copy number variations in these GBM tissues as indicated by Danussi's report [22]. Instead, we paid attention on its function in GBM cells. Our data support that PINX1 promoted cell proliferation, since both loss- and gain-of-function studies led to the consistent consequences. After revealing its function, we set to find out the direct miRNA that target PINX1, since many studies have reported that miRNAs play a significant role in the development of human cancers [23]. We found that miR-627 could direct impact on the luciferase activity of PINX1 reporter, its mRNA and protein expression. Additionally, the decreased expression of miR-627 negatively correlated with PINX1 in GBM tissues. Furthermore, our experi-

ments further confirmed the role of miR-627 as an tumor suppressor in GBM cells. We showed that overexpression of miR-627 significantly suppressed GBM cell proliferation. Hence, our work is the first report to link tumour-suppressive miR-627 with oncogene PINX1 in GBM cells.

Taken together, our work revealed that PINX1 was upregulated and functioned as an oncogene by promoting cell proliferation in GBM. We identified that miR-627 could directly target PINX1 and regulate its expression, and served as a potent tumor suppressor in GBM. Further studies on the molecular mechanisms of PINX1 in tumorigenesis are still needed.

Disclosure of conflict of interest

None.

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References

- [1] Zhou XZ and Lu KP. The Pin2/TRF1-interacting protein PinX1 is a potent telomerase inhibitor. *Cell* 2001; 107: 347-359.
- [2] Liao C, Zhao MJ, Zhao J, Jia D, Song H and Li ZP. Over-expression of LPTS-L in hepatocellular carcinoma cell line SMMC-7721 induces crisis. *World J Gastroenterol* 2002; 8: 1050-1052.
- [3] Lin J and Blackburn EH. Nucleolar protein PinX1p regulates telomerase by sequestering its protein catalytic subunit in an inactive complex lacking telomerase RNA. *Genes Dev* 2004; 18: 387-396.
- [4] Sun C, Wu Z, Jia F, Wang Y, Li T and Zhao M. Identification of zebrafish LPTS: a gene with similarities to human LPTS/PinX1 that inhibits telomerase activity. *Gene* 2008; 420: 90-98.
- [5] Kondo T, Oue N, Mitani Y, Kuniyasu H, Noguchi T, Kuraoka K, Nakayama H and Yasui W. Loss of heterozygosity and histone hypoacetylation of the PINX1 gene are associated with reduced expression in gastric carcinoma. *Oncogene* 2005; 24: 157-164.
- [6] Ma Y, Wu L, Liu C, Xu L, Li D and Li JC. The correlation of genetic instability of PINX1 gene to clinico-pathological features of gastric cancer in the Chinese population. *J Cancer Res Clin Oncol* 2009; 135: 431-437.
- [7] Shi R, Zhao Z, Zhou H, Wei M, Ma WL, Zhou JY and Tan WL. Reduced expression of PinX1 correlates to progressive features in patients with prostate cancer. *Cancer Cell Int* 2014; 14: 46.
- [8] Cai MY, Zhang B, He WP, Yang GF, Rao HL, Rao ZY, Wu QL, Guan XY, Kung HF, Zeng YX and Xie D. Decreased expression of PinX1 protein is correlated with tumor development and is a new independent poor prognostic factor in ovarian carcinoma. *Cancer Sci* 2010; 101: 1543-1549.
- [9] Liu JY, Qian D, He LR, Li YH, Liao YJ, Mai SJ, Tian XP, Liu YH, Zhang JX, Kung HF, Zeng YX, Zhou FJ and Xie D. PinX1 suppresses bladder urothelial carcinoma cell proliferation via the inhibition of telomerase activity and p16/cyclin D1 pathway. *Mol Cancer* 2013; 12: 148.
- [10] Zhang R, Zhao J, Wang X, Wang LL, Xu J and Song C. PinX1 without the G-patch motif suppresses proliferation, induces senescence, but does not inhibit telomerase activity in colorectal cancer SW480 cells. *Oncol Rep* 2014; 32: 286-292.
- [11] Qian D, Zhang B, He LR, Cai MY, Mai SJ, Liao YJ, Liu YH, Lin MC, Bian XW, Zeng YX, Huang JJ, Kung HF and Xie D. The telomere/telomerase binding factor PinX1 is a new target to improve the radiotherapy effect of oesophageal squamous cell carcinomas. *J Pathol* 2013; 229: 765-774.
- [12] Tian XP, Qian D, He LR, Huang H, Mai SJ, Li CP, Huang XX, Cai MY, Liao YJ, Kung HF, Zeng YX and Xie D. The telomere/telomerase binding factor PinX1 regulates paclitaxel sensitivity depending on spindle assembly checkpoint in human cervical squamous cell carcinomas. *Cancer Lett* 2014; 353: 104-114.
- [13] Ohgaki H and Kleihues P. Epidemiology and etiology of gliomas. *Acta Neuropathol* 2005; 109: 93-108.
- [14] Tzadok S, Beery E, Israeli M, Uziel O, Lahav M, Fenig E, Gil-Ad I, Weizman A and Nordenberg J. In vitro novel combinations of psychotropics and anti-cancer modalities in U87 human glioblastoma cells. *Int J Oncol* 2010; 37: 1043-1051.
- [15] Fiorenzo P, Mongiardi MP, Dimitri D, Cozzolino M, Ferri A, Montano N, Trevisi G, Maira G, Battistini L, Falchetti ML, Levi A and Pallini R. HIF1-positive and HIF1-negative glioblastoma cells compete in vitro but cooperate in tumor growth in vivo. *Int J Oncol* 2010; 36: 785-791.
- [16] Wilson TA, Karajannis MA and Harter DH. Glioblastoma multiforme: state of the art and future therapeutics. *Surg Neurol Int* 2014; 5: 64.
- [17] Bai J, Chen YS, Mei PJ, Liu QH, Du Y and Zheng JN. PinX1 is up-regulated and associated with poor patients' survival in gliomas. *Int J Clin Exp Pathol* 2015; 8: 6952-6959.
- [18] Lewis BP, Burge CB and Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 2005; 120: 15-20.
- [19] Zhou XZ, Huang P, Shi R, Lee TH, Lu G, Zhang Z, Bronson R and Lu KP. The telomerase inhibitor PinX1 is a major haploinsufficient tumor suppressor essential for chromosome stability in mice. *J Clin Invest* 2011; 121: 1266-1282.
- [20] Zhang B, Qian D, Ma HH, Jin R, Yang PX, Cai MY, Liu YH, Liao YJ, Deng HX, Mai SJ, Zhang H, Zeng YX, Lin MC, Kung HF, Xie D and Huang JJ. Anthracyclines disrupt telomere maintenance by telomerase through inducing PinX1 ubiquitination and degradation. *Oncogene* 2012; 31: 1-12.
- [21] Zhang B, Bai YX, Ma HH, Feng F, Jin R, Wang ZL, Lin J, Sun SP, Yang P, Wang XX, Huang PT, Huang CF, Peng Y, Chen YC, Kung HF and Huang JJ. Silencing PinX1 compromises telomere length maintenance as well as tumorigenicity in telomerase-positive human cancer cells. *Cancer Res* 2009; 69: 75-83.

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- [22] Danussi C, Akavia UD, Niola F, Jovic A, Lasorella A, Pe'er D and Iavarone A. RHPN2 drives mesenchymal transformation in malignant glioma by triggering RhoA activation. *Cancer Res* 2013; 73: 5140-5150.
- [23] Mutlu M, Raza U, Saatci O, Eyupoglu E, Yurdusev E and Sahin O. miR-200c: a versatile watchdog in cancer progression, EMT, and drug resistance. *J Mol Med (Berl)* 2016; 94: 629-644.