**Original Article**

**PinX1 is up-regulated and associated with poor patients’ survival in gliomas**

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**Abstract:** PinX1, a conserved nuclear protein, could maintain telomere integrity and plays an important role in regulating telomerase activity. It has been reported that the expression of PinX1 is down-regulated in some cancer and associated with cancer prognosis. However, the value of PinX1 in gliomas has not been studied. In this study, two independent retrospective gliomas cohorts with the corresponding gliomas tissue microarrays (TMAs) were established to detect the expression level of PinX1 and the correlation of PinX1 expression with the clinicopathological features and the patients’ survival. Compared with non-cancerous brain tissues, PinX1 protein levels were remarkably up-regulated in gliomas \( (P = 0.001) \), and further increased from benign gliomas tissues to malignant gliomas tissues \( (P = 0.090) \). Moreover, high PinX1 expression was significantly positively associated with gliomas WHO grade in the training set \( (P = 0.019) \) and the validation set \( (P = 0.037) \). High PinX1 expression significantly correlated with a worse 5-year overall \( (P = 0.016) \) and disease-specific survival \( (P = 0.026) \). Simultaneously, the multivariate COX regression analysis showed that PinX1 was an independent unfavorable prognostic factor for 5-year overall survival (hazard ratio (HR) = 2.078, \( P = 0.015 \)) and disease-specific survival (HR = 2.429, \( P = 0.012 \)) after adjusting with age, sex and WHO grade in gliomas. In conclusion, PinX1 expression may serve as a prognostic and predictive biomarker for gliomas.

**Keywords:** PinX1, gliomas, tissue microarray, WHO grade, prognosis

**Introduction**

Although the incidence rate of human malignant tumors in brain and central nervous system is low, there will be still nearly 22,810 new cases diagnosed world widely in 2014 [1]. Among all the types of cancers in brain and central nervous system, gliomas are the most common malignancies and cause most of deaths [2]. Especially, glioblastoma multiform (GBM), one special type of gliomas, is the most aggressive one on account of its fast growth and frequently spreading to nearby brain tissue. A series of powerful treatments have been made for the GBM patients, however, due to the inadequate surgery and the inapproachable chemotherapeutic drugs when crossing the blood-brain barrier, the GBM has become the most incurable and lethal cancer with a survival of less than 12 months [3]. Now, accumulating evidences have indicated that the changed gene expressions will help us to discover the effective biomarkers for gliomas. Therefore, it is urgent for us to study and find these biomarkers which could provide targets for predicting the prognosis and the therapy of gliomas.

Telomere is composed of repetitive nucleotide sequences and exists at the terminal of every linear chromosome to prevent it from fusion with the neighboring chromosomes [4]. In mammalian cells, six specialized telomeric proteins, including Pot1, TPP1, TIN2, TRF1, TRF2 and Rap1, form the shelterin which is capped at the telomeric DNA to maintain the telomere integrity and homeostasis [5]. In the normal cells, telomere length is precisely controlled and becomes
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significantly shortened telomere lengths, meanwhile the knockdown of PinX1 expression suppresses cell proliferation and enhances cell apoptosis in the telomerase-positive cancer cells [18]. Now, there is still no study to conduct on the expression and prognostic functions of PinX1 in gliomas so far. Therefore, in this study, we used the two retrospective gliomas patient cohorts with the tissues microarrays (TMAs) to investigate the expression of PinX1 and the association of PinX1 expression with clinicopathological features and outcome in gliomas.

Materials and methods

Patients and samples

This study was performed under a protocol approved by the Institutional Review Boards of Xuzhou Medical College, and all examinations were performed after obtaining written informed consents. Two independent retrospective gliomas patient cohorts, called the training set and validation set, were used to investigate the role of PinX1 in this study. In the training cohort, as our previous report [19], 123 gliomas patients with Grades I-II and 68 gliomas patients with Grades III-IV were recruited. Simultaneously, in order to validate the findings in the training set, we recruited another 216 gliomas patients with Grades I-II and 176 gliomas patients with Grades III-IV to build the validation set. Patients entering the validation cohort were followed up from the date of surgery to the date of death or the last follow-up. The tissues were obtained from The Affiliated Hospital of Xuzhou Medical College. Exclusion criteria were patients with preoperative chemotherapy or radiotherapy. Due to the lost samples during antigen retrieval or samples with no tumor cells present in the core, ultimately, 191 cases in the training cohort and 392 cases in the validation cohort was used to analysis the PinX1 expression (Table 1).

### Table 1. Relationship between PinX1 staining and clinicopathological characteristics of the individuals in two cohorts of glioma patients

<table>
<thead>
<tr>
<th>Variables</th>
<th>Training cohort (191 cases)</th>
<th>Validation cohort (392 cases)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low (%)</td>
<td>High (%)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 47 years</td>
<td>38 (40.9)</td>
<td>55 (59.1)</td>
</tr>
<tr>
<td>≥ 47 years</td>
<td>37 (37.8)</td>
<td>61 (62.2)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>42 (35.6)</td>
<td>76 (64.4)</td>
</tr>
<tr>
<td>Female</td>
<td>33 (45.2)</td>
<td>40 (54.8)</td>
</tr>
<tr>
<td>WHO Grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>17 (60.7)</td>
<td>11 (39.3)</td>
</tr>
<tr>
<td>II</td>
<td>37 (38.9)</td>
<td>58 (61.1)</td>
</tr>
<tr>
<td>III</td>
<td>7 (21.2)</td>
<td>26 (78.8)</td>
</tr>
<tr>
<td>IV</td>
<td>14 (40.0)</td>
<td>21 (60.0)</td>
</tr>
</tbody>
</table>

*χ² test.
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Construction of TMA and immunohistochemistry of TMA

The glioma TMA of the training set was obtained from Shanxi Chaoying Biotechnology (Xi’an, Shanxi, China), which included array 1.0 mm diameter dots of 8 normal brain tissues, 8 tumor adjacent normal brain tissues, and 191 gliomas tissues. The gliomas TMA of the validation set was created in National Engineering Center for Biochip, Shanghai, China. The duplicate 1.0 mm diameter cores of 392 gliomas tissues was punched from the paraffin block and assigned in the glass slides.

The immunohistochemistry of TMAs was carried out as described previously [19]. The anti-PinX1 antibody (1:50 dilution, Novus Biologicals, Littleton, USA) was used for primary antibody
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Table 2. Univariate Cox proportional regression analysis on 5-year overall and disease-specific survival of 136 glioma patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>Overall survival</th>
<th>Disease-specific survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hazard ratio</td>
<td>95% CI</td>
</tr>
<tr>
<td>PinX1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>1.000</td>
<td>0.019</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤47 years</td>
<td>1.000</td>
<td>0.443</td>
</tr>
<tr>
<td>&gt;47 years</td>
<td>0.864</td>
<td>0.594-1.225</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1.000</td>
<td>0.392</td>
</tr>
<tr>
<td>Female</td>
<td>0.810</td>
<td>0.500-1.312</td>
</tr>
<tr>
<td>WHO Grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-II</td>
<td>1.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

*P values are from Log-rank test. CI: confidence interval.

incubation at 4°C overnight. The slide without primary antibody incubation was used as negative control.

Evaluation of immunostaining

The evaluation of PinX1 staining was blindly and independently examined by two pathologists by applying a semi-quantitative immunoreactivity score (IRS), as reported before [20], and the consensus was asked to reach for every core. IRS was the multiplying scores of staining intensity and the percentage of positive cells. The intensity of PinX1 immunostaining was 0 (negative), 1 (weak), 2 (moderate) and 3 (strong), the percentage of immunoreactive cells was 1 (0-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%). Based on the IRS, PinX1 was classified as low (IRS: 0-4) and high (IRS: 6-12) expression.

Statistical analysis

For TMA, statistical analysis was performed with SPSS 20.0 software (SPSS, Inc, Chicago, IL). Differences in IRS for PinX1 staining in normal brain tissues, tumor adjacent normal brain tissues and gliomas tissues were assessed by the Fisher’s exact test. The association between PinX1 staining and the clinicopathologic parameters of the glioma patients, including age at diagnosis, sex and WHO grade, was evaluated by X² test. The Kaplan-Meier method and log-rank test were used to evaluate the correlation between PinX1 expression and patient survival. Cox regression model was used for multivariate analysis. Differences were considered significant when P < 0.05.

Results

PinX1 expression is up-regulated in gliomas

In order to investigate the expression level of PinX1 in gliomas, the immunohistochemical staining of a TMA which included 8 normal brain tissues, 8 tumor adjacent normal brain tissues and 191 gliomas tissues was used. We found that PinX1 staining was mainly located in the nucleus (Figure 1A-D). PinX1 was highly expressed in 1 of 8 (12.5%) normal brain tissues, 2 of 8 (25%) tumor adjacent normal brain tissues, 69 of 123 (56%) benign gliomas tissues and 47 of 68 (69%) malignant gliomas tissues (Figure 2). The expression of PinX1 was notably increased in the gliomas tissues when compared with the normal brain tissues (P = 0.001, Fisher’s exact test), and further increased in malignant gliomas tissues when compared with benign gliomas tissues (P = 0.090, Fisher’s exact test). The most remarkable difference of PinX1 expression was between the normal brain tissues and malignant gliomas tissues (P = 0.003, Fisher’s exact test).

PinX1 expression was significantly associated with WHO grade

The clinicopathologic characteristics of the training cohort and the validation cohort of glioma biopsies were summarized in Table 1. Samples with IRS 0-4 and IRS 6-12 were classified as low and high expression of PinX1. As shown in Table 1, chi-square analysis revealed that the expression of PinX1 in the gliomas tissues was significantly positively associated with the WHO grade in the training set (P = 0.019) (Table 1). These findings were confirmed...
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Increased PinX1 expression correlates with poor patient survival

To further study whether increased PinX1 staining in glioma patients correlates with a worse prognosis, Kaplan-Meier survival curves were constructed using 5-year overall or disease-specific cumulative survival to compare the patients with high PinX1 staining to those with low PinX1 staining (n = 136, follow-up time, 60 months). Our data revealed that high PinX1 staining correlated with both worse overall and disease-specific cumulative survival rate dropped from 61.8% in patients with low PinX1 expression to 46.7% in those with high PinX1 expression.

**PinX1 serves as an independent molecular prognostic indicator for gliomas**

Moreover, we examined whether PinX1 expression was an independent prognostic factor for gliomas. We performed a univariate Cox regression analysis including PinX1 expression, age, gender and WHO grade to study the effects of PinX1 on patient survival in gliomas. The univariate Cox regression analysis showed that PinX1 expression was an independent prognostic marker for glioma patients overall survival (hazard ratio, 1.981; 95% CI, 1.118-3.511; P = 0.019; Table 2), and disease-specific survival
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(hazard ratio, 1.741; 95% CI, 1.230-2.755; \( P = 0.024; \text{Table 2} \)). In multivariate Cox regression analysis, we found that PinX1 expression was also an independent prognostic marker for 5-year overall survival (hazard ratio, 2.078; 95% CI, 1.153-3.744; \( P = 0.015; \text{Table 3} \)) and disease-specific survival (hazard ratio, 2.429; 95% CI, 1.222-3.630; \( P = 0.012; \text{Table 3} \)) after adjusting with age, sex and WHO grade in gliomas.

Discussion

Various data have indicated that PinX1 functionally inhibits telomerase activity and shortens telomeres [11]. The expression of PinX1 is significantly reduced in some human cancers and associated with the poor outcome of the cancer patients. Moreover, the suppressive roles of PinX1 have also been confirmed in the heterozygous mice (PinX1\(^{-/-}\)) which have the increased opportunities for breast, lung, and gastrointestinal carcinomas development [21]. In some human cancer cell, PinX1 has been proved as a tumor suppressor to decrease cell proliferation and metastasis, such as colorectal cancer [16], bladder cancer [17], gastric cancer [22]. But, several studies have also showed the different and even contradictory roles of PinX1 in telomerase activity and cancer cells proliferation [8, 18], which reminds us that the function of PinX1 is depended on the types of cancers. However, the exact role of PinX1 in the gliomas is still unclear. Therefore, in this study, we used the two independent gliomas TMA with the responding clinical and survival data to determine the prognostic role of PinX1 in the gliomas.

On the basis of 191 gliomas tissues with different WHO grades, 8 gliomas adjacent normal brain tissues and 8 normal brain tissues, the result of TMA immunohistochemistry indicated that the expression of PinX1 was significantly up-regulated in gliomas tissues when compared with the normal brain tissues. Now some studies have focused on finding the reasons of differential PinX1 expression in cancer, and the attempt has been done to establish the relationship of the PinX1 somatic mutation with some carcinomas [23], but there was a rare somatic mutation reported. Moreover, the study about medulloblastomas has been reported that PinX1 has no somatic mutation, and meanwhile the expression of PinX1 is not been suppressed in cancer [24]. These data may indicate that the role of PinX1 is specific in nervous tumors in comparison with some other cancer.

Next, in these two independent gliomas patient cohorts, we found that PinX1 expression was further increased in malignant gliomas tissues when compared with the benign gliomas tissues. The PinX1 expression was positive association with the gliomas WHO grades. These results indicated that PinX1 expression may function in increasing the malignancy of gliomas. Furthermore, interestingly, in the survival analysis, our results revealed that the high expression of PinX1 was significantly correlated with the poor 5-year overall and disease-specific survival for the gliomas patients, which is contradictory to the studies on gastric cancer [12, 13], prostate cancer [14], ovarian cancer [15]. The univariate and multivariate Cox proportional regression analysis declared that PinX1 expression was an independent unfavorable prognostic factor for predicting both 5-year overall and disease-specific survival. Our results demonstrated that the function and role of PinX1 was heterogeneous in gliomas, which may from another side prove the role of PinX1 depending on the type of cancer.

Recently, it has been reported that directly targeting to PinX1 could augment the anti-cancer effects. Zhang B et al. have showed that anthracyclines can down-regulate PinX1 to interrupt telomere maintenance by telomerase, which strongly enhances the cancer cells’ sensitivity to chemotherapy. Tian X et al. have indicated that knockdown of PinX1 dramatically enhances paclitaxel cytotoxicity, whereas the reestablishment of PinX1 levels substantially reduces the paclitaxel-induced killing effect [25]. Moreover, in the gliomas, Long L et al. have demonstrated that PinX1-siRNA combined with doxorubicin enhances the inhibition effect on the glioma cell growth [26]. These studies combined with our data indicated that the PinX1 may be an effective biomarker and target for the gliomas prognosis and treatment, however, the selection bias was still unavoidable from retrospective studies and the exact role of PinX1 in gliomas remains to require further investigations. Therefore, the basic researches and the prospective clinical studies about
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PinX1 are further asked to validate whether PinX1 is a useful biomarker for gliomas patients.

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Disclosure of conflict of interest

None.

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