Original Article
Upregulation of PAX3 confers a poor prognosis in Chinese glioblastoma patients with IDH1 wild type by increasing cell proliferation and migration

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Abstract: Glioblastoma multiforme (GBM) is a common lethal brain tumor. Genes involved in the pathogenesis of GBMs may have diagnostic value or be used as important prognostic factors. We have profiled whole genome mRNA expression array in 119 GBM patients and 5 normal brain tissues and transcriptome sequencing in 98 GBM patients from the Chinese Glioma Genome Atlas (CGGA) cohort to identify prognostic markers for GBM patients. Multivariate Cox proportional hazard regression analysis (p<0.01) and Significance Analysis of Microarrays (SAM, FDR<5%) were performed to identify prognostic genes in the GBMs group. Meanwhile, gene expression profile data of 196 GBMs from The Cancer Genome Atlas (TCGA) network was utilized to validate our results. Only genes significant in both CGGA and TCGA databases were chosen for further analysis. Kaplan-Meier survival analysis was performed to estimate overall survival of all GBM patients. PAX3 was identified as the most significantly prognostic gene using the above methods. Overexpression of PAX3 indicated poor survival in the 183 CGGA IDH1 wild type GBM patients but not in those of TCGA IDH1 wild type GBM patients. Prognostic value of PAX3 overexpression in GBMs was not interfered by clinicopathological features. Functional annotation of mRNA profiling and transcriptome data in GBMs from the CGGA and TCGA cohorts showed that patients with higher PAX3 expression tended to have a higher expression of proliferation associated genes. Knocking down the expression of PAX3 in U87 glioblastoma cells with PAX3 siRNA resulted in significant decrease in GBM cell proliferation and migration. The present study suggests that overexpression of PAX3 could be a marker with poor prognosis in IDH1 wild type GBMs by increasing cancer cell proliferation and migration.

Keywords: IDH1 wild type glioblastoma, PAX3, prognosis, proliferation, migration.

Introduction
Glioblastoma multiforme (GBM) is the most malignant brain tumor of the central nervous system in adult. The World Health Organization (WHO) in the group of diffusely infiltrating astrocytomas (WHO grade IV), which account for 50% all primary brain glioma, classifies GBM [1-3]. The new WHO classification of GBM has been refined and now includes the IDH1/2 mutation [4]. Even with improved treatment modalities, GBMs have a uniformly poor prognosis, with a median overall survival (OS) of only 14-16 months [5-7]. GBMs are heterogeneous tumors despite its seemingly uniform pathology, which are characterized by considerable variability in biological behavior and gives rise to significantly different prognoses and responses to treatment [8]. The molecular markers specific to malignancy have been reported to have diagnostic value or as an important prognostic factors [9-11]. However, the number of clinically relevant markers for GBM still remains limited. Therefore, searching more of prognostic and diagnostic molecular biomarkers for GBMs to provide direction for clinic practices is urgent.
The paired box3 (PAX3) transcription factor is a member of the paired box family that is highly conserved throughout phylogeny. PAX3 plays key roles during embryogenesis and cancer epigenesis [12-19]. PAX3 gene is first expressed in early embryos, in neural crest precursor cells [20-23], and is an important factor for delamination of the neural crest during embryogenesis [24], which regulates myogenesis, melanogenesis and neurogenesis [25]. The expression of PAX3 can be detected in the limb muscle, neural crest, and neural tube [26]. PAX3 is known to regulate melanocytes, melanoma and glioma cells proliferation and migration [12, 27]. However, it is not known whether PAX3 continues to be associated with prognosis of GBM with or without IDH mutation.

In this study, we compared the gene expression of PAX3 between 119 glioblastoma tumors and 5 non-tumor brain tissues using mRNA expression array analysis and validated our results with 196 TCGA GBMs expression data and 98 CGGA transcriptome sequencing data to identify the correlation between PAX3 expression and patient survival. Meanwhile, we analyzed the effect of PAX3 on the progression of GBMs with or without IDH1 mutation.

Materials and methods

Tissue samples

The Research Ethics Committee of Beijing Tiantan Hospital approved the study protocol. Informed consent was obtained from all patients. A total of 217 glioblastoma multiforme (GBM) samples were obtained from the Chinese Glioma Genome Atlas (CGGA) database. Fresh tumor tissue samples were immediately snap-frozen in liquid nitrogen after resection and stored in liquid nitrogen until processing. Two histopathologists confirmed all tumors independently. Clinicopathological information was obtained from medical records of the Chinese Glioma Genome Atlas (CGGA) Database. Five normal brain tissues from non-tumor patients were collected as controls.

mRNA expression assays

A total of 119 whole fresh frozen GBM samples and 5 normal brain tissues obtained from CGGA underwent mRNA expression profiling using the Agilent Whole Human Genome Oligo Microarray, which is in a 4*44k slide format: each block represents more than 41,000 unique human genes and transcripts. Total RNA was extracted from 20-30 mg of the all frozen tumor samples and normal samples using a Total RNA Isolation Kit (Ambion, Austin, TX) according to the manufacturer's protocol. RNA concentrations were measured with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Houston, TX). cDNA preparation, labeling, hybridization and scanning were performed according to the manufacturer's protocol as previously reported [28].

Expression array analysis

In order to maximize the power to detect genes involved in progression of GBM, we compared mRNA expression in 119 GBM samples with the 5 normal brains from CGGA. The microarray data normalization and filtering was performed using the GeneSpring GX 11.0 (Agilent Technologies). Only genes with expression levels marked as present or marginal in all of the chips (blocks) passed through the quality filtering. Then, measurements were set to less than 0.01, and the chip data was normalized to the 50th percentile of the intensity of each chip. Multivariate Cox proportional hazard regression analysis was performed to estimate the significance of the association for each mRNA expression with overall survival. Those with significant association with survival (p<0.01) were selected for further analysis. Genes that significantly up or down regulated were identified using Significance Analysis of Microarrays (SAM). Analysis parameters (Delta) were set to result in FDR<5%. Meanwhile, we used 196 TCGA GBM mRNA expression data to verify our results. Similarly, methods including Multivariate Cox proportional hazard regression analysis and Significance Analysis of Microarrays (SAM) were used to identify genes that its expression altered compared with normal brain tissues and correlated with patients' survival. Genes significant in both databases were chosen for further research. The Kaplan-Meier method, analyzed the association between gene levels and patients' survival. PAX3 was identified the most significantly prognostic gene.

RNA sequencing

RNA sequencing was performed for 98 GBM samples from CGGA database. Standard QC criteria was applied to raw data to remove all
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The reads that fit any of the following parameters: 1) the reads that aligned to adaptors or primers with no more than two mismatches. 2) the reads with more than 10% unknown bases (N bases). 3) the reads with more than 50% of low quality bases (quality value ≤ 5) in one read. Clean data were subsequently mapped to the reference human genome (UCSC hg19) with Spliced Transcripts Alignment to a Reference (STAR) [29].

**GSVA with PAX3 expression**

For proliferation functional annotation, Gene Set Variation Analysis (GSVA) with PAX3 expression was analyzed by GSVA package [30] of R. Gene lists of proliferation and anti-proliferation were from the Gene Ontology (GO) term GO: 0008283 and GO: 0008285.

**Cell culture and siRNA analysis**

Human glioblastoma cell line U87 was obtained from the Department of Neurosurgery, Laboratory of Neuro-Oncology, Tianjin Medical University General Hospital. The cells were cultured in DMEM (HyClone, USA), supplemented with 10% fetal bovine serum (HyClone) and kept in a humidified 5% CO₂ atmosphere at 37°C. Cells were grown on sterilized culture dishes and were passaged every 2 days with 0.25% trypsin (Invitrogen). PAX3 siRNA (Dharmacon) was transfected into the cells using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. Cells transfected with negative siRNA were used as control interference group.

**RT-PCR**

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DMSO (SERVA) for 30 min. Absorbance at a wavelength of 450 nm was determined with a microplate reader (Bio-Tek ELX800, USA).

**In vitro migration assay**

Cell migration assay were performed using a 24-well Trans-well chambers (Corning, USA) with cell culture inserts (pore-size: 8μm). At 48 h post-transfection, cells were harvested and $5 \times 10^4$ cells were transferred to the upper compartment of the transwell insert in 200 μl DMEM medium without serum. In the lower chamber, 500 μL DMEM with 10% FBS was added as a chemoattractant. After the cells were incubated for 24 h at 37°C in a 5% CO$_2$ atmosphere, the insert was washed with PBS, and cells on the top surface of the insert were removed with a cotton swab. Cells adhering to the lower surface were fixed with methanol, colored using 0.1% crystal violet and counted under a microscope in five predetermined fields. All assays were independently repeated at least thrice.

**Statistical analysis**

Statistical analysis was performed using SPSS13.0. Data were expressed as mean ± standard deviation (SD). Statistical comparisons between groups were performed using ANOVA and a non-parametric test. The survival analysis was performed using log-rank test in GraphPad Prism 5. Patients in the database were divided into high PAX3 and low PAX3 groups based on their median expression value. The GSVA was performed with the PAX3 expression going from low to high. Differences were considered statistically significant at P<0.05.

**Results**

**Expression arrays**

Totally, 119 GBM samples and 5 normal brain tissues from CGGA database underwent mRNA expression profiling. In order to detect genes involved in progression of GBMs, we used Multivariate Cox proportional hazard regression analysis (p<0.01) and Significance Analysis of Microarrays (SAM) (FDR<5%) methods to screen significantly prognostic genes. Meanwhile, 196 GBMs expression data were downloaded from TCGA database to verify our results. We used the same method analyzed the TCGA data. 6 genes were identified significant alteration in both databases, when analyzed with Multivariate Cox proportional hazard regression analysis and SAM, such as increased PAX3, C13orf18, SPATA6 expression and decreased DCTD, NRN1, STARD8 expression. The association between gene expression level and patients’ survival were further analyzed by Kaplan-Meier method. It is showed that high expression of PAX3 and C13orf18 and low expression of STARD8 indicated poor survival of GBM patients. PAX3 were overexpressed in GBM and was chosen for further analysis because it was the most significant gene for causing malignant progression (Table 1).

**Overexpression of PAX3 was associated with poor prognosis of GBMs with IDH1 wild type**

After PAX3 was identified as a prognostic gene in pGBM patients in both CGGA and TCGA databases, we further analyzed the association between PAX3 expression level and patients’ survival. Kaplan-Meier plots for 119 pGBM profiles showed high expression of PAX3 highly correlated with poor prognosis by comparing to its low expression (median overall survival, 386.0 vs 506.0 days; p=0.0038, log-rank test; Figure 1A). Similar trends were identified from GBM patients with IDH1 wild type rather than those with mutant IDH1 (median overall survival, 387.0 vs 481.0 days; p=0.0373, log-rank test, Figure 1B). We then performed whole-transcriptome sequencing on 98 GBM patients from CGGA database for validation. Both patients harbored GBM (median overall survival, 358.0 vs 660.0 days; p=0.0227, log-rank test, Figure 1C) and patients harbored IDH1 wild type GBM (median overall survival, 347.0 vs 432.0 days; p=0.0422, log-rank test, Figure 1D) with over-expressed PAX3 demonstrated shorter overall survival time than those with lower-expressed PAX3. Kaplan-Meier plots for 196 TCGA pGBM profiles also showed that high expression of PAX3 indicated poor overall survival by comparing to its low expression.

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**Table 1. Genes overexpressed in GBMs compared to normal brain tissues**

<table>
<thead>
<tr>
<th>Rank</th>
<th>Gene</th>
<th>Change</th>
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<th>FDR</th>
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<tbody>
<tr>
<td></td>
<td>Upregulation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>PAX3*</td>
<td>3.8</td>
<td>&lt;11.1%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>C13orf18</td>
<td>2.0</td>
<td>&lt;11.1%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Downregulation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>STARD8</td>
<td>0.8</td>
<td>&lt;15.0%</td>
<td></td>
</tr>
</tbody>
</table>

*Gene chosen for further analysis.
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(mean overall survival, 406.1 vs 547.6 days; p=0.0193, log-rank test; Figure 1E). We did not find the association between PAX3 expression and patients overall survival in patients with IDH1 wild type GBM from TCGA set. Both in CGGA and TCGA database, PAX3 expression was not related to overall survival time of patients with mutant IDH1 GBM (Figure 1F).

Patients with higher risk score tended to have a higher expression of proliferation associated genes and a lower expression of anti-proliferation associated genes (Figure 2A and 2B). To investigate further oncogenic characteristics of PAX3, we performed RNA sequencing on 98 GBM samples and found enhanced enrichment of proliferation signatures expression in GBM.

Prognostic value of PAX3 overexpression in GBM were not interfered by clinicopathological features

In order to exclude the interference of clinicopathological features on prognostic value of PAX3 overexpression in GBMs, patients were divided into two groups: PAX3 low expression group and PAX3 high expression group, based on the expression profile data in CGGA and TCGA database, respectively. The gender, age at diagnosis, Karnofsky Performance Status (KPS) score, extent of resection and chemotherapy which were not significantly different (P≥0.05, one-way ANOVA or Student’s t-test, respectively) between PAX3 low expression group. The results suggested that the prognostic value of PAX3 overexpression in GBM were not interfered by clinicopathological features.

Proliferation functional annotation of the two groups with different risk scores

As we found that the function of the PAX3 gene, which were able to divide patients into two subgroups with different prognosis, were mainly associated with proliferation, we further performed GSVA with the risk score going from low to high. The gene lists of proliferation and anti-proliferation were from the GO terms GO:0008283 and GO:0008285.
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patients with PAX3 over-expression. (Figure 2C). That might be an explanation of the different prognosis of the two groups divided by risk score.

**Downregulation of PAX3 inhibited cellular proliferation in U87 cells**

To investigate whether PAX3 impacts on the proliferation of glioblastoma cells, we used human GBM cell lines U87 to study. PAX3 siRNA was transfected into U87 cells and negative siRNA was transfected as control groups. Transcriptional levels of PAX3 in U87 cell were assessed by RT-PCR (Figure 3A). Protein expression levels of PAX3 were analyzed by western blot method (Figure 3B). The results showed that PAX3 siRNA could efficiently knockdown the expression of PAX3. Thus, PAX3 siRNA was applied in subsequent MTT assay. Knocking down of PAX3 expression by specific siRNA markedly inhibited the proliferative capability of U87 cells (Figure 3C).

**Downregulation of PAX3 inhibited cellular migration in U87 cells**

After confirming that PAX3 siRNA could efficiently knockdown the expression of PAX3 in U87 cells (Figure 3), we investigated the impact of PAX3 on U87 cell migration. PAX3 siRNA and negative siRNA were transfected into U87 cells. 48 h later, cells were transferred to Trans-well chambers. After 24 h incubation, the percentage of migrated cells was significantly less in PAX3 siRNA treated groups compared to control group (negative siRNA) (P<0.05) (Figure 4).

**Discussion**

GBM is a seemingly uniform pathology with variety heterogeneous clone tumor, which associated with great variability in terms of response to treatment and clinical outcome [8]. They were classified according to the 4th edition of the World Health Organization (WHO) classification published in 2007 [8]. In most recent years, integrated genomic analysis of gliomas has identified IDH mutation as the key mutation in gliomagenesis. Therefore, the updating 4th WHO classification of tumors of the central nervous system (CNS) stratifies the group of “diffuse astrocytic and oligodendroglioma tumors” according to the occurrence of IDH mutation as a new entity [4].

Genes involved in the pathogenesis of GBMs may have diagnostic value or as an important prognostic factor. Recent reports based on gene expression profiling from patient tumor samples to identify prognostic or predictive genes is becoming increasingly common in clinical practice, and has identified genes associated with tumor progression and patients survival [31, 32]. In the present study, we compared the mRNA expression profile of 119 patients with PAX3 over-expression. (Figure 2C). That might be an explanation of the different prognosis of the two groups divided by risk score.
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pGBM samples with 5 normal brains from CGGA database to identify prognostic gene in GBMs. 196 TCGA expression data of GBMs were used to confirm our results. We finally identified 2 genes were significantly overexpressed in the GBM group in both database, as PAX3 and C13orf18. PAX3 gene was chosen for further analysis, as it was the most significant gene in GBM group for causing malignant progression. (Table 1).

PAX3 plays important roles during embryogenesis and cancer epigenesis [11-18]. Deregulated expression of PAX3 has been identified in several human malignancies, such as melanocytes, rhabdomyosarcoma, and Ewing’s sarcoma [26]. Moreover, PAX3 has been detected in other malignancies, such as breast cancer and small cell lung [33, 34]. High expression of PAX3 is associated with degree of malignancy, rapid cell proliferation, and poor patient survival in different tumors. This suggests that overexpression of PAX3 may correlate with pathologic staging of GBM and have significant value as a biomarker in GBM patients. Although PAX3 is known to facilitate glioma cells proliferation and migration [12, 27], it is not known whether PAX3 continues to control pathways of proliferation and migration in GBM with or without IDH1 mutation and then contributes to GBM progression.

In the present study, we identified the overexpression of PAX3 in 217 CGGA GBM samples and 196 TCGA GBM samples compared to 5 normal brain tissues, and the overexpression of PAX3 indicated poor prognosis in GBM patients. And high expression of PAX3 in GBM has only recently been confirmed [35]. We further confirmed that prognostic value of PAX3 overexpression in GBM was not interfered by clinicopathological features. Overexpression of PAX3 is a significant poor prognosis marker in GBMs. In addition, we have found that PAX3 overexpression indicated shorter overall survival time only in patients with IDH wild type GBM rather than those with IDH1 mutant. Similar trends have been identified in both microarray and sequencing set from CGGA database. Unexpectedly, PAX3 expression was not associated with overall survival time in neither IDH1 mutant or wild type GBM in TCGA dataset, indicating a race heterogeneity between Caucasian and Chinese population. It is suggested that PAX3 distincts GBM patients as a potential therapeutic target for Chinese patients harbored IDH1 wild type GBM.

Other proteins might modulate the transcriptional activity of PAX3 proteins. For example, PAX3 transcription can be modulated by FGF2-STAT3 signaling in the melanocytes [36]. Myc family of transcription factors may modulate Pax3 expression in vivo [20]. In the current study, the GBM cell line, U87 cell was selected for researching on the function of PAX3 in GBMs. RNAi is involved in post-transcriptional gene silencing (PTGS) and does not affect replication or transcription of DNA sequences. Its function is also highly specific, effective, and successful [37]. The current research demonstrated that the expression of PAX3 in U87 cells in the interference group decreased significantly comparing to that in the control group.

Figure 3. PAX3 konckdown inhibits proliferation in U87 cells. Different treatments included Neg siRNA group (control) and PAX3 siRNA transfected group. A. RT-PCR assay demonstrated PAX3 RNA expression in U87 glioblastoma cells transduced with Neg siRNA and PAX3 siRNA. At 48 h post transfection, U87 cells were harvested and total RNA was extracted using TRizole. GAPDH served as a loading control. B. Western blot showing protein expression levels in Neg siRNA and PAX3 siRNA treatments. U87 cells were harvested at 48 h after transfection and whole cell lysates prepared using RIPA buffer. β-tubulin served as a loading control. C. Effect of PAX3 knockdown on the proliferation of U87 cells was measured by MTT assay. A decrease in absorbance at 450 nm was observed.
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An association between PAX3 expression level and cell proliferation has been observed in neuronal cells [38], schwann cell [39] and myoblasts [40] in vitro. PAX3 antisense oligonucleotides inhibited the growth of the alveolar rhabdomyosarcoma cell line Rh30 and melanoma cell lines [41, 42]. In the current study, GSVA showed patients with higher PAX3 expression tended to have a higher expression of proliferation associated genes and lower expression of anti-proliferation associated ones in GBMs from the CGGA and TCGA. MTT analysis was performed to detect the effect of PAX3 on the growth and proliferation of glioblastoma cell. Our data showed that PAX3 siRNA effectively inhibits proliferation of U87 cells (Figure 3C). This indicated that RNAi inhibition of PAX3 expression reduced the proliferation of U87 cells, which suggested that PAX3 might play an important role in the progression of GBMs by promoting GBM cell proliferation.

Pax3 was essential for migration of limb muscle precursors and melanocyte precursors [40, 42, 43]. In the current study, the Transwell migrate test showed that the cell migration in the interference group was significantly lower than that in the control group. It indicated that RNAi inhibition of PAX3 expression reduced the migration of U87 cells, which suggest that PAX3 may modulate the migration of GBM cell during the process of GBMs.

The present study has enhanced our knowledge about the effects of PAX3 on GBMs. The biological functions of PAX3 found in this study provided a mechanistic basis for the pathological and clinical observations. High expression of PAX3 indicates poor prognostic of IDH1 wild type GBM patients in Chinese population. The result was not interfered by clinicopathological features. We provided compelling evidence that decreased expression of PAX3 inhibits cell proliferation and migration. The data suggests overexpression of PAX3 confers a poor prognosis in GBMs through increasing GBM cell proliferation and migration. The molecular mechanism underlying the effects of PAX3 on GBM cell proliferation and migration need to be further analyzed.

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Disclosure of conflict of interest

None.

Abbreviations

PAX3, Paired box3; GBM, Glioblastoma multiforme; CGGA, The Chinese Glioma Genome Atlas; TCGA, The Cancer Genome Atlas; SAM, Significance Analysis of Microarrays; FDR, False Discovery Rate; OS, Overall survival.
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References


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[38] Reeves FC, Burdge GC, Fredericks WJ, Rauser FJ and Lillycrop KA. Induction of antisense PAX-3 expression leads to the rapid morphological differentiation of neuronal cells and an altered response to the mitogenic growth factor bFGF. J Cell Sci 1999; 112 (Pt 2): 253-261.


