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Human positive coactivator 4 (PC4) is involved in the progression and prognosis of astrocytoma

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ABSTRACT

Astrocytoma is the most common primary brain tumor and it is associated with poor prognosis. Accumulating evidences suggest that certain molecular abnormalities or genetic mutations are associated with its progression and prognosis. Human transcriptional coactivator 4 (PC4), originally identified as a transcriptional coactivator then as a DNA replication and repair factor has been shown to be involved in chromatin organization. Recently, it is reported to function both as tumor suppressor and promoter depending on the cellular settings. In the present study, we for the first time demonstrated that the expression of PC4 in astrocytoma was upregulated as assessed by western blot and immunohistochemical staining. Moreover, elevated PC4 expression was strongly correlated with the progression of astrocytoma. Furthermore, high PC4 expression was also associated with poor overall survival. Finally, in vitro study demonstrated that siRNA mediated PC4 downregulation significantly inhibited the proliferation and invasiveness of human glioma cells. These results suggested that PC4 might play a role in human astrocytoma progression and may be used as a novel indicator for the prognosis of astrocytoma patient.

1. Introduction

Glioma is the most frequent primary brain tumor in adults, which by far accounts for 70% of adult malignant primary brain tumors, with yearly incidence of 6 in 100,000 [1]. Despite of optimal management with maximal surgical resection (when feasible) [2], radiotherapy [3], chemotherapy [4] and concomitant therapy, the prognosis for newly diagnosed malignant glioma remains poor [5] with a median survival time of 12 to 15 months for patients with glioblastoma and 2 to 5 years for patients with anaplastic glioma. The etiology of glioma is largely unknown. Although ionizing radiation [6] and genetic mutation [7] are recognized as risk factors by some studies, the molecular mechanism of its development and progression remains poorly understood. Hence, it is of great clinical value to further investigate the molecular mechanisms underlying progression of glioma and to identify novel prognostic factors and therapeutic targets.

Human transcriptional coactivator 4 (PC4) is a multifunctional protein which plays diverse roles in cellular processes, including transcription, replication, repair and heterochromatinization [8–11]. PC4 has also been linked to tumor development and progression as a putative tumor suppressor, which can inhibit self-repression of AP2 in a ras-transformed cell line [12]. The tumor suppressive activity of PC4 is also manifested through its ability to enhance p53 function [13,14]. However, in contrast to serving as a potential tumor suppressor, PC4 has recently been recognized as a putative cancer promoter. Upregulated PC4 expression was reported in several cancers, including non-small cell lung cancer (NSCLC) [15], invasive intraductal papillary mucinous neoplasm of the pancreas [16], primary colorectal carcinomas [17] and small cell lung cancer (SCLC) cell lines [18]. Moreover, the knockdown of PC4 significantly inhibited the growth of cancer cells in NSCLC [15]. However, whether PC4 is expressed and involved in glioma is not known.

In the present study, we investigated the potential role of PC4 in human glioma. Western blot and immunohistochemical staining were used to analyze the expression of PC4 in human astrocytoma. The relationship between PC4 expression and astrocytoma progression were also assessed. Kaplan–Meier analysis and log-rank test were performed to calculate the effect of PC4 expression on patients' survival time. Finally, the effect of PC4 downregulation on the proliferation and invasion of glioma cells was further investigated through in vitro study.

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2. Materials and methods

2.1. Patient selection and tissue sample collection

Patients with astrocytoma who underwent complete primary resection from March 2004 to June 2005 at the Institute of Neurosurgery of Tangdu Hospital were included in this study. Patients who have previous malignancy, second primary tumor, or those who received preoperative radiotherapy and/or chemotherapy were excluded. All 122 patients included in the study were diagnosed histologically by at least two experienced pathologists independently. A total of 122 (65 men and 57 women) paraffin-embedded tumor samples were collected. In 75 cases, tissues adjacent to tumor were collected as control. Age of the patients ranged from 9 to 76 years (median, 42.5 ± 15.6 years). There were 21 (17.2%) grade I, 43 (35.2%) grade II, 36 (29.5%) grade III, and 22 (18.1%) grade IV diseases (WHO grades). 3 additional frozen samples with paired adjacent tissues were also obtained for western blot analysis. For the experimental use of the samples, informed consent was obtained from the patients. The study was approved by the Ethics Committee of Tangdu Hospital, the Fourth Military Medical University.

2.2. Western blot

3 frozen glioma samples with paired adjacent tissues were homogenized in 400 μl of RIPA buffer (0.05 M Tris–HCl [pH 7.4], 0.15 M NaCl, 0.25% deoxycholic acid, 1% Nonidet P-40, and 1 mM EDTA) containing protease inhibitors (1%, Sigma St. Louis, MO, USA). The protein concentration was measured with a BioRad protein assay kit (BioRad, Hercules, CA, USA). 50 μg of total proteins was separated by SDS-PAGE and blotted onto PVDF membranes (Millipore, Bedford, MA). Overnight incubation was carried out at 4 °C in TBS and Tween-20 (TBS-T; 20 mM Tris–HCl [pH 8.0],150 mM NaCl, and 0.05% Tween-20) containing primary antibodies against PC4 (1:500 Santa Cruz Biotechnology, CA), and followed by incubation with HRP-labeled goat anti-rabbit second antibody (1:2000; Santa Cruz Biotechnology, CA) for 1 h at 37 °C. Bands were visualized by a chemiluminescence (ECL) system (Pierce, Rockford, IL, USA).

2.3. Immunohistochemistry

All tissue sections (5 μm) were dewaxed, rehydrated and incubated in 3% hydrogen peroxide for 15 min at room temperature to quench the endogenous peroxidase activity. Antigens were retrieved by incubating in 10 mM sodium citrate (pH 6.0) at 95–100 °C for 15 min. The nonspecific binding was blocked by incubating the sections with 0.1 M PBS containing 0.6% Triton X-100 and 10% normal goat serum (NGS) for 1 h at room temperature. Then the sections were incubated at 4 °C overnight with primary human PC4 antibody (1:500; Santa Cruz Biotechnology, CA), followed by incubation with biotinylated second antibody and then the streptavidin–biotin peroxidase complex (Sigma, St. Louis, MO, USA). After incubation in a DAB kit, slides were lightly counterstained with hematoxylin, dehydrated through a graded ethanol series, cleared with xylene, and mounted with neutral balsam. The expression of PC4 was evaluated by staining intensity and the extent in a specimen as described previously [19]. The intensity of staining was graded 0–3 (0 = no staining; 1 = weak staining; 2 = moderate staining; and 3 = strong staining), and the extent of staining was scored as follow: 0 (no positive tumor cells), 1 (<10% positive tumor cells), 2 (10–35% positive tumor cells), 3 (35–70% positive tumor cells) and 4 (>70% positive tumor cells). The final quantification of each staining was obtained by multiplying the 2 scores. High PC4 expression was identified by the final quantification score of ≥6, while low PC4 expression with score of ≤4. The slides were analyzed by 2 pathologists independently.

2.4. RNA interference assay

RNA interference assay was performed as previously reported [20]. The siRNA sequences targeting human PC4 were as follows: forward, 5′-r[ACAGAGCAGCAGCAGCAGA]dTT-3′; reverse, 5′-r[UCUGCUGCUG CUGCUCUGU]dTT-3′. The scramble siRNA as negative control: forward, 5′-r[UUCUCCGAACGUGUCACGU]dTT-3′; reverse, 5′-r[ACGUGACACGUU CGGAGAA]dTT-3′ (GenePharma, Shanghai, China). The transfection of siRNAs into glioma cells was done using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols. Cells were transfected with siRNA for 48 h before functional assays were carried out.

2.5. Cell proliferation assay

Cell proliferation was evaluated by methyl thiazolyl tetrazolium (MTT) assay. 48 h after transfection, U251 or U87-MG cells were harvested, seeded into 96-well plates with a concentration of 3000 cells per well, and incubated in a 5% CO_2 incubator at 37 °C. Cell viability was determined every 24 h. At each time point, 10 μl of 5 mg/ml MTT (Sigma, St Louis, USA) was added, and incubation was continued for 4 h. At the end of the incubation, the medium was removed carefully and 100 μl of DMSO was added. The absorbance was measured at 570 nm by a microplate reader (Bio-Rad, Hercules, CA).

2.6. Invasion assay

Cell invasion was assessed using the matrigel invasion system. Transwell chambers with a pore size of 8 μm (Corning, NY, USA) were coated with 100 μg growth factor reduced matrigel (BD Biosciences) according to manufacturers' instructions. 48 h after transfection, cells were seeded into the upper chamber at a density of 3.0 × 104 cells/well in 200 μl of serum-free medium. The lower chamber was filled with medium containing 10% serum as chemoattractant. Following incubation at 37 °C and 5% CO_2 for 24 h, cells remaining on the upper side of the membrane were removed with cotton swabs. Those on the bottom side of the membrane were fixed with methanol and stained with crystal violet. Cells were counted and images were captured in ten randomly selected fields under a microscope at 200× magnification (Nikon Corporation, Tokyo, Japan).

2.7. Statistical analysis

All data were analyzed by SPSS 16.0. Chi-square test and Fisher exact test were used to compare the levels of PC4 expression with both different groups and various clinicopathologic parameters. Bivariate correlations between two independent variables were analyzed by calculating the Spearman's correlation coefficients. Survival analysis was performed using the Kaplan–Meier method and compared by the log-rank test. Prognostic relevance was evaluated by multivariate Cox regression analysis. For the in vitro cell study, comparisons among all groups were performed using one-way analysis of variance (ANOVA). $p < 0.05$ was considered as statistically significant.

3. Results

3.1. PC4 protein expression is increased in astrocytoma

To investigate the expression of PC4 protein in astrocytoma, comparative analysis was conducted on three pairs of matched astrocytoma tissues and their adjacent non-cancerous counterparts using western blot. As shown in Fig. 1A, the expression of PC4 protein was significantly upregulated in all three human primary astrocytoma samples compared with their paired adjacent non-cancerous tissues. To further confirm these observations, immunohistochemical staining was performed in 122 paraffin-embedded astrocytoma samples and 75 paired adjacent