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Original Articles

Protein kinase D2 promotes the proliferation of glioma cells by regulating Golgi phosphoprotein 3

Xiuping Zhou [a,b,*,1], Pengfei Xue [c,1], Minglin Yang [c,1], Hengliang Shi [a,b], Dong Lu [a,b], Zhaohao Wang [c], Qiong Shi [a,b], Jinxia Hu [a,b], Shao Xie [a,b], Wenjian Zhan [a,b], Rutong Yu [a,b,**]

[a] Brain Hospital, Affiliated Hospital of Xuzhou Medical College, Xuzhou, Jiangsu, China
[b] Insititute of Nervous System Diseases, Xuzhou Medical College, Xuzhou, Jiangsu, China
[c] The Graduate School, Xuzhou Medical College, Xuzhou, Jiangsu, China

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ABSTRACT

Protein kinase D2 (PKD2) has been demonstrated to promote tumorigenesis in many types of cancers. However, how PKD2 regulates cancer cell growth is largely unknown. In this study, we found that over-expression of PKD2 promoted glioma cell growth but down-regulation of PKD2 inhibited it. Further investigation indicated that PKD2 down-regulation decreased the protein level of Golgi phosphoprotein 3(GOLPH3) as well as p-AKT level. On the contrary, over-expression of PKD2 increased the protein level of GOLPH3 and p-AKT. In addition, GOLPH3 exhibited similar effect on glioma cell growth to that of PKD2. Importantly, GOLPH3 down-regulation partially abolished glioma cell proliferation induced by PKD2 over-expression, while over-expression of GOLPH3 also partially rescued the inhibition effect of PKD2 down-regulation on glioma cell growth. Interestingly, the level of PKD2 and GOLPH3 significantly increased and was positively correlated in a cohort of glioma patients, as well as in patients from TCGA database. Taken together, these results reveal that PKD2 promotes glioma cell proliferation by regulating GOLPH3 and then AKT activation. Our findings indicate that both PKD2 and GOLPH3 play important roles in the progression of human gliomas and PKD2-GOLPH3-AKT signaling pathway might be a potential glioma therapeutic target.

Introduction

Malignant glioma is the most common brain tumor with the median survival time of only 12–15 months, despite the fact that the patients are usually treated with a combined approach of surgery, chemotherapy and radiation therapy [1,2]. It is critically necessary to discover its molecular mechanism and find specific molecular targets to cure glioma.

The Protein kinase D (PKD) family belongs to the calcium-/calmodulin-dependent protein kinase superfamily and comprises three mammalian isoforms: PKD1, PKD2 and PKD3 [3–5]. Physiologically, PKDs associate with the cytoplasmic surface of golgi membranes to regulate the fission of vesicles that carry protein and lipid cargo from the TGN to the plasma membrane [6]. Recent studies also show that PKD plays an irreplaceable role in regulating many cellular activities like cardiac hypertrophy and remodeling [7], neuronal polarity formation [8], immune regulation [9], endocrine [10], and so on. In addition to the physiological function, PKD2, one of the most important PKD hypotype, is found to be involved in the proliferation of several kinds of cancers, such as glioblastoma [11], colorectal cancer [12], pancreatic cancer [13], breast cancer [14], prostate cancer [15], and gastric cancer [16] in the past few years. It is shown that PKD2 could promote prostate cancer cell invasion through p65 NF-κB and HDAC1-mediated expression and activation of uPA [15]. Although there are some studies which elaborate the role of PKD2 in the development of cancer, the mechanism that PKD2 promotes glioma tumorigenesis is still largely unknown.

Golgi phosphoprotein 3 (GOLPH3), a membrane protein with a molecular weight of 34 kD [17,18], is mainly located in the trans-Golgi network [19]. GOLPH3 has the following functions: promoting vesicle transportation from Golgi to the cell membrane, participating the glycosylation of protein, maintaining Golgi structure, and transporting the Golgi proteins [20–23]. However, more and more evidence indicate that GOLPH3 is also a key regulator in the development of cancers. Scott et al. firstly demonstrates that *GOLPH3* is a new oncogene, which is commonly targeted for amplification in human cancer and is capable of modulating the response to a

Abbreviations: PKD2, Protein kinase D2; GOLPH3, Golgi phosphoprotein 3; TGN, trans-golgi network; PI4KIIIβ, phosphatidylinositol-4 kinase IIIβ; YB1, Y-box-binding protein 1.

* Corresponding author. Tel.: +86 0516 85587335; fax: +86 0516 85587335.
E-mail address: xpzhou@xzmc.edu.cn (X. Zhou).
** Corresponding author. Tel.: +86 0516 85802367.
E-mail address: yu.rutong@163.com (R. Yu).
[1] These authors contributed equally to this work

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clinical drug, rapamycin [24]. High expression level of GOLPH3 has been reported in hepatocellular carcinoma, glioma, renal cell carcinoma and epithelial ovarian carcinoma, further supporting the tumor oncogenic function of GOLPH3 [25–28]. It has been shown that GOLPH3 promotes the development and progression of cancer via several downstream molecules such as PI3K-AKT-mTOR pathway [24], Rho A [29], FOXO1 [30], sialylation [31] and YB1 [32]. However, these studies mostly focused on the downstream signaling of GOLPH3. So far, the upstream regulatory molecules of GOLPH3 have not been reported.

In summary, PKD2 and GOLPH3 hold many similar functions in cancer biology. However, the relationship between PKD2 and GOLPH3 in cancer development is unknown. It is shown that PKD family could phosphorylate PI4KIIIβ at the Golgi complex [33]. PI4KIIIβ regulation largely determines the sites and amounts of PtdIns(4)P produced within Golgi membranes. Moreover, PKD regulates the activity of PI4K and several PtdIns(4)P effectors that control sphingolipid and sterol content of Golgi membranes [34]. Vps74/GOLPH3 is the newly identified PtdIns4P effector and PtdIns4P recognition by Vps74/GOLPH3 links PtdIns 4-kinase signaling to retrograde Golgi trafficking [34]. In addition, PtdIns(4)P is required for GOLPH3 localization to Golgi and depletion of PtdIns(4)P phenocopies knockdown of GOLPH3 [35]. Thus, we speculate that PKD2 may regulate GOLPH3. In this study, we desired to elucidate that PKD2 is an upstream regulatory molecule of GOLPH3 in regulating AKT activity in the process of glioma cell proliferation.

Materials and methods

Cell culture

The human glioma U251 and U87 cell lines were purchased from Shanghai Cell bank, Type Culture Collection Committee, Chinese Academy of Sciences. The cells were cultured in Dulbecco's modified Eagle's medium and F-12 (DMEM/F-12) (Invitrogen) supplemented with 10% fetal bovine serum (Sijiqing Biological Engineering Materials Co.) and grown in a humidified incubator with 5% CO_2 at 37 °C.

Small interfering RNA transfection

Knocking down of PKD2 and GOLPH3 was carried out using siRNA Oligos (Shanghai GenePharma Co, Ltd, Shanghai, China). The sets of siRNA duplexes are as follows:

siNC Sense: 5′-UUCUCCGAACGUGUCACGUTT-3′
Anti-sense:5′-ACGUGACACGUUCGGAGAATT-3′
PKD2-siA Sense: 5′-AAUGACCUUAACUGCCACGUCCCGG-3′
Anti-sense:5′-CCGGGACGUGGCAGUUAAGGUCAUU-3′
PKD2-siB Sense: 5′-CCUGAGUGUGGCUUCUACGGCCUUU-3′
Anti-sense: 5′-AAAGGCCGUAGAAGCCACACUCAGG-3′
GOLPH3-si792 Sense: 5′-GUUAAGAAAUGUACGGGAATT-3′
Anti-sense:5′-UUACAGAUUACCUUUCUUGTT-3′

Transfection of siRNA oligos with Lipofectamine™ 2000 (Invitrogen) was carried out as instructed by the manufacturer. One day before transfection, 2×10^5 cells were plated in six-well plate such that they will be 30~50% confluent at the time of transfection. One hundred picomoles of siRNA duplex and 5 μL Lipofectamine 2000 reagent was diluted in 250 μL Opti-MEM, respectively. After 5 min incubation, the diluted oligo and Lipofectamine 2000 reagent were combined and incubated for 20 min at room temperature. Thereafter, the oligo-Lipofectamine 2000 complex was evenly added into cells. The plate was mixed gently by rocking back and forth for several times. All siRNA silencing experiments were performed three times independently with each duplex.

Immnuofluoresence

The transfected cells were fixed with 4% paraformaldehyde for 20 min at 4 °C. After being washed with phosphate buffer saline (PBS), the cells were permeabilized with 0.1% Triton X-100 for 10 min and blocked with 1% bovine serum albumin/PBS for 10 min at room temperature. Then, the cells were incubated with the primary antibody diluted with 1% bovine serum albumin/PBS for 1 h at room temperature, followed by washing and incubating with Alexa-conjugated secondary antibody. The nuclei were labeled with DAPI. Washed cells were embedded in glycerin and photographed by Olympus IX-71 inverted microscope (Olympus Corporation, Shinjuku, Tokyo, Japan).

EdU incorporation assay

Transfected cells were seeded into 96-well plates at 7×10^3 cells per well. Twenty-four hours later, the cells were exposed to 50 μM of 5-ethynyl-20-deoxyuridine (EdU, Ribobio, Guangzhou, China) for additional 2 h at 37 °C. Then, the cells were fixed with 4% paraformaldehyde for 20 min and treated with 0.5% Triton X-100 for another 20 min at room temperature. After being washed with PBS for five times, the cells were reacted with 100 μL of 1 × Apollo® reaction cocktail for 30 min. Thereafter, the DNA contents of cells were stained with 100 uL of Hoechst 33342 (5 μg/mL) for 20 min and visualized under a fluorescent microscope (Olympus, IX71).

Cell viability assay

Cell viability was measured with the Cell Counting Kit-8 (CCK-8, Beyotime, China) for three times independently with each duplex. The single cell suspension were dispensed in a 96 well plate, cultured for 24 h, and then transfected with siRNA or plasmids for up to 48 h. CCK-8 reagent (10 μL) was added in each well and incubated for another 2 h. Then the absorbance at 450 nm wavelength (OD450) was measured using a microplate reader (Bio-Tek, Synergy 2).

Western blotting analysis

The cells were lysed in RIPA lysis buffer (containing 2 μg/mL Aprotinin; 100 μM Leupeptin; 1 μM Pepstatin; 200 μM PMSF; 1 mM Benzamidine; 1 mM DTT; 1 mM NaF; 1 mM Na_3VO_4) for 20 min on ice with occasional vortex. Protein concentrations of the cell lysates were measured using the BCA Protein Assay Kit (Thermo Scientific). Equal amount of proteins was separated on a 10% or 12% SDS-PAGE and transferred electrophoretically to the PVDF membranes. The membrane was blocked with 3% BSA or 5% Skimmed milk in Tris-buffered saline with tween (TBST) for 2 h at room temperature. The blots were incubated with antibodies against PKD2 (1:500, Abcam, Cambridge, UK), p-AKT (1:200, Santa Cruz, CA, USA), AKT (1:500, Santa Cruz), β-actin (1:3000, Millipore, Billerica, MA, USA), GOLPH3 (1:1000, Abcam), GFP (1:500, Santa Cruz), myc (1:1000, Millipore) at 4 °C over night. After being washed with TBST, the membranes were incubated with peroxidase-conjugated secondary antibodies (dilution 1:4000) for 1 h at room temperature. The bands were visualized with chemiluminescence detection kit (Pierce, Rockford, IL, USA) and the result was analyzed by Image ProPlus Software. The relative amount of proteins was determined by normalizing the densitometry value of interest to that of the internal loading control.

Immunohistochemistry

Paraffin-embedded pathological sections were provided by the Department of pathology, Affiliated Hospital of Xuzhou Medical College (7 of non-tumor brain tissue, 4 of GradeII glioma tissue, 6 of Grade III glioma tissue, 5 of Grade IV glioma tissue). Written informed consent was obtained from each patient and the study was approved by the Research Ethics Committee. Immunohistochemical staining was performed using the protocol supplied by the S-P. immunohistochemistry kit (Zhongshan Goldenbridge Biotech CO.). The sections were fixed with 4% paraformaldehyde and blocked with 10% goat serum. Then, the sections were incubated with PKD2 or GOLPH3 antibody (PBS as blank) followed by HRP-conjugated goat anti-mouse IgG (Invitrogen). The reaction was developed by 3, 3′-diaminobenzidine (DAB) chromogenic reagent (Zhongshan Goldenbridge Biotech CO.). The sections were counterstained with hematoxylin to stain the nucleus and dehydrated by incubation in increasing concentrations of alcohol, followed by 100% xylene, before coverslips were mounted onto the slides with neutral gum. The photos were collected under an Olympus IX-71 inverted microscope (Olympus).

RT-PCR

Total RNA was isolated from the transfected cells and the cDNA was synthesized using reverse transcription reagents (Roche) according to the manufacturer's protocol. All PCR primers were synthesized by Sangon Biotech (Shanghai, China). The primer sequences were as follows. β-actin forward: 5′-CATGTACGTTGCT ATCCAGGC-3′; Reverse: 5′-CTCCTTAATGTCACGCACGAT-3′; GOLPH3 forward: 5′-TGTAAG- TCAGATGCTCCAACAGG-3′, Reverse: 5′-TCACCCATTTGTCAAGAACGG-3′. The product was 249 bp and 316 bp, respectively.

Statistical analysis

The results are representative of experiments repeated at least three times and quantitative data were expressed as means ± SEM. Statistical comparisons were performed using Student's *t*-test with two tails or ANOVA for multiple comparisons. *P* values less than 0.05 were considered statistically significant ($^*P < 0.05$). All statistical analyses were performed using Office Excel 2003 (Microsoft Corporation) or SPSS software (SPSS version 16.0).