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Over expression of PPP2R2C inhibits human glioma cells growth through the suppression of mTOR pathway

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ABSTRACT

PPP2R2C encodes a gamma isoform of the subunit B55 subfamily, which is a regulatory subunit of Protein phosphatase type 2A (PP2A). Our study shows that PPP2R2C is downregulated in glioma cells and human brain cancer patient samples. Overexpression of PPP2R2C inhibited cancer cell proliferation both in vitro and in vivo through the suppression of the activity of S6K in the mTOR pathway. Moreover, exogenous expression of PPP2R2C promoted the formation of a complex with the PP2A-C subunit to further enhance the binding of PP2A-C with S6K. Our results suggest that PPP2R2C is a potential tumor suppressor gene in human brain cancers. This study will provide novel insight into the development of therapeutic strategies in the treatment of human brain tumors.

Structured summary of protein interactions:
PP2A-C physically interacts with **B55 Gamma** by anti bait coimmunoprecipitation (View interaction)
B55 Gamma physically interacts with **PP2A-C** by anti bait coimmunoprecipitation (View interaction)

1. Introduction

Protein phosphatase type 2A (PP2A) is a serine/threonine phosphatase consisting a family of holoenzyme complexes involved cellular behaviors [1]. In general environment, the holoenzyme is a core dimer, composed of the structural A and catalytic C subunits, and a regulatory B subunit that associates with a variety of substrates [1,2]. As a serine/threonine phosphatase, PP2A can dephosphorylate many signaling enzymes, and inhibition of this phosphatase can affect cellular activities such as growth, differentiation, and apoptosis [2]. Among the targets of PP2A are proteins of oncogenic signaling cascades, such as Ras, Raf, MEK, and AKT [3–6]. This suggests that specific PP2A holoenzymes play a role in cellular proliferation and oncogenic transformation [2]. It has been reported that inhibition of PP2A can cause variety of tumor type's growth and metastasis [7–10]. Therefore, understanding the precise mechanisms how PP2A is involved in the regulation of these different signaling cascades and its role during oncogenic transformation requires the identifying specific regulatory subunit involved in the progress.

The regulatory B subunit can modulate the activity of the PP2A, by targeting a wide range of PP2A substrates [11]. To date, four unrelated families of B subunits have identified: B/B55/PR55/PPP2R2, B' /B56/PR61/PPP2R5, B/PR72/PPP2R3, and Striatin with at least 16 members in these subfamilies [12]. The variable PP2A B subunits are targeted by a number of oncogenes, such as Ras and AKT [3,6]. PPP2R2C encodes a gamma isoform of the regulatory subunit B55 subfamily-B55 Gamma. Currently, the precise functions of PPP2R2C are still under investigating. It has been reported that PPP2R2C had a unique expression pattern in mouse brain which suggested a role in learning and memory [13]. They found PPP2R2C was correlated to intellectual disability (ID). Another study reported that B55 Gamma was an inhibitor of c-Jun NH2-terminal kinase (JNK) activation by UV irradiation. They showed that PR55 gamma bound with c-SRC and modulated the phosphorylation of serine 12 of c-SRC, a residue which is required for JNK activation by c-SRC [14].

Although PP2A is a well-studied cancer suppressor protein, whether PPP2R2C which encodes a B regulatory subunit plays a role in tumor progression has not been well studied yet. A recent study by Bluemn et al. demonstrated that loss of PPP2R2C promotes androgen ligand depletion-resistant prostate cancer growth, which was independent of AR-mediated transcriptional programs [15], indicating PPP2R2C might be a tumor suppressor gene in prostate cancers.

In this study, we reported a novel tumor suppression function of B55 Gamma which encoded by PPP2R2C in human glioma cells and brain tumors. PPP2R2C was down regulated in glioma cell lines and brain tumor patient samples and overexpression of PPP2R2C inhibited the cancer cell proliferation through the suppression of mTOR pathway. This study will provide novel insight into the tumor suppression function of PPP2R2C and encourage the design and testing

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of approaches targeting this protein in the therapeutics of human brain tumor patient.

2. Materials and methods

2.1. Cell lines and patient samples

The human glioma cell lines (U87, U138, NHA, U118 LN18, U251, T406 and U343) were obtained from ATCC. Cells were grown adherently in DMEM media supplemented with 10% fetal bovine serum (FBS; Sigma–Aldrich Chemical Company) and 1% penicillin–streptomycin (GIBCO BRL, Grand Island, NY) and maintained in a humidified incubator containing 5% CO_2 at 37 °C. Cells were routinely grown in 100-mm plastic tissue culture dishes (Nunc, Roskilde, Denmark) and harvested with a solution of trypsin–EDTA while in a logarithmic phase of growth. Cells were maintained in these culture conditions for all experiments.

All primary Human GBM patient specimens were obtained from patients undergoing surgery for brain tumor during 2009–2012 at the Shanghai Renji Hospital Cancer Center and stored in liquid nitrogen until analysis. Human normal brain whole tissue lysates (#1, #2 and #3) were purchased from OriGene (CP565621, CP565723 and CP565732). Human normal brain whole tissue lysates (#4, #5 and #6) were obtained from Shanghai Renji Hospital Cancer Center. Human primary astrocytes were purchased from http://www.scinecelloneline.com. All patients provided written informed consent. The study was approved by the Ethics Committee of the Renji Hospital Cancer Center, Shanghai Jiaotong University Medical College, Shanghai, China.

2.2. Vectors

Vector containing Myc-DDK-tagged ORF clone of Homo sapiens protein phosphatase 2, regulatory subunit B, gamma (PPP2R2C), transcript variant 1 was purchased from Origene (RC224154). Wild-type S6K and kinase active S6K were purchased from Addgene (#26610, #8988: pRK7-HA-S6K1-F5A-E389).

2.3. Antibodies and siRNA

B55Gamma (Sigma SAB1406287); mTOR pathway and substrate antibody sampler kit (Cell signaling #9862 & #9964); β-actin (Cell signaling #4967); PP2A-A (Cell signaling #2041); PP2A-C (Cell signaling #2038); siPPP2R2C (EHU059901 Sigma MISSION® siRNA human PPP2R2C).

2.4. Gene expression database analysis

mRNA expression profiling of PPP2R2C in human tissues was analyzed through EMBL-EBI website: http://www.ebi.ac.uk/.

2.5. Immunohistochemistry staining

Immunohistochemistry (IHC) staining of tumor and normal tissue sections was done with an ABC kit using DAB (3,3′-Diaminobenzine) detection. Purified anti-B55 Gamma antibody (Sigma SAB1406287) was identified to stain specifically on paraffin embedded sections. All normal human brain tissue and GBM patient samples were from the Biological Resource Centre of Renji Hospital, Shanghai Jiaotong University Medical College.

2.6. Cell viability assay

Cell viability was measured using CellTiter-Glo™ Luminescent Cell Viability Assay (Promega). In brief, 1000 cells were seeded in 96-well plate and the cell number was measured following manufacturer's instructions.

2.7. Co-immunoprecipitation

For Co-immunoprecipitation (Co-IP), cells were harvested, washed with PBS, then lysed in IP buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 5 mM EDTA [pH 8.0], 0.5% NP40, 1 mM PMSF, complete Protease Inhibitor Cocktail tablet supplemented with phosphatase inhibitors [1 mM Na_3VO_4, 20 mM NaF, 0.1 mM b-glycerophosphate, 20 mM sodium pyrophosphate]). Lysates were then cleared by centrifugation (14000 rpm, 10 min) and protein concentration in supernatants was determined with a BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of total protein (2MG) were used for IP as described. Primary antibody and control IgG were added into lysates for incubation overnight at 4 °C cold room. After 2 h of incubation with agarose G plus beads, beads were washed and eluted then elutes were subjected to 10% SDS–PAGE gel electrophoreses.

2.8. Anchorage-independent colony formation assay

For glioma cells foci formation assay, 500 cells were seeded on 10 cm dish with regular cell culture medium. Cells without or with exogenous PPP2R2C were grown for 2 weeks and the surviving colonies were stained with gentian violet after methanol fixation, and visible colonies (>50 cells) were counted. Colonies from randomly-selected image areas of three replicate wells were enumerated.

2.9. Western blotting analysis

Cells were lysed with RIPA buffer (50 mM Tris–HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 5 mM EDTA and HALT Protease Inhibitor Cocktail (Pierce, Thermo Fisher Scientific, Rockford, IL). Protein concentrations were determined with BCA Protein Assay kit (Pierce). Unless stated otherwise, 30 μg of protein lysates were separated in 10% SDS–PAGE gels and blotted onto nitrocellulose membranes (Macherey–Nagel, Düren, Germany) and probed with the antibodies listed in the above. Signals were detected using ECL substrate solution and recorded with Fusion-SL 3500 WL image acquisition system (Vilber Lourmat, Marne-la-Vallée, France).

2.10. siRNA knockdown of PPP2R2C

siRNA experiments were performed in 24-well plates using lipofectamine 2000 transfection conditions. Human PPP2R2C-targeting siRNA and negative control siRNA were purchased from Sigma–Aldrich. 24 h after transfection at 400 nM for both negative control and siPPP2R2C, cell proliferation were assessed by adding Cell Titer-Glo and measuring luminescence.

2.11. Xenograft tumor growth of glioma cells in nude mice

The female athymice BALB/c nude mice (5–8 week-old) were housed in the Biological Resource Centre of Renji Hospital, Shanghai Jiaotong University Medical College. Mice were implanted subcutaneously in both sides of flank with 3×10^6 glioma cells without or with transfection of PPP2R2C, respectively. Tumor progress was monitored by tumor size measurements at every other day.

2.12. Statistical data analysis

All data were analyzed using GraphPad Prism 5.04. Quantitative data are shown as mean ± S.E.M. (standard error of the mean)