R280T mutation of p53 gene promotes proliferation of human glioma cells through GSK-3β/PTEN pathway

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HIGHLIGHTS

▶ R280T is first identified in glioma cell line according to IARC TP53 database.
▶ GSK-3β/PTEN pathway may be associated with R280T-mediated growth of SWO-38 cell.
▶ The findings provide valuable insights for uncontrolled growth of glioma cells.

ABSTRACT

p53 mutation is associated with “gain-of-function” capabilities of human cancers. We aim to identify p53 mutations in human glioma cells and to explore the potential mechanism for mutant p53-promoted cellular growth. Whole genomic DNA was isolated from SWO-38, a human glioma cell line and amplified for the region of exons 5, 6, and 8 in p53 gene using polymerase chain reaction (PCR). By means of direct sequencing of PCR products and alignment analysis using BLAST database, a mutation of G to C transition at codon 280 of p53 exon 8 (AGA → ACA), i.e. R280T was detected in SWO-38 cells. Knockdown of R280T mutant p53 by RNA interference inhibited the GSK-3β/PTEN associated cell proliferation, and PI3K/Akt but not Wnt/β-catenin signaling pathway was involved in this process. Furthermore, depletion or overexpression of PTEN alone did not affect cell proliferation and cell cycle, implicating the impairment of PTEN function in SWO-38 cells. However, knockdown of both PTEN and p53 mutation could significantly rescue the p53 depletion-mediated growth inhibition, suggesting that the R280T mutation in glioma may promote the proliferation through an underlying mechanism related to PTEN. Our observations indicate that the R280T mutation of p53 regulates the proliferation of human glioma cells related to the GSK-3β/PTEN pathway. These findings provide valuable insights for better understanding the molecular mechanism of uncontrolled growth of glioma cells.

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1. Introduction

The p53 signaling pathway, has been suggested to be an essential molecular regulator in regulating glioma oncogenesis [2]. However, the mechanisms underlying tumorigenesis and development of human malignant gliomas remain unclear. The p53 tumor suppressor prevents the propagation of cells with unstable genomes by halting the cell cycle in G1 phase or initiating apoptosis or proliferative arrest [26]. It is suggested that p53 is one of the most frequently mutated tumor suppressors in human gliomas [22,25]. p53 mutations may not only have lost tumor-suppressive functions but may have also acquired pro-oncogenic properties, which are known as “gain-of-function” activities [9,11,17,24,30].

Mutant p53 (e.g. A220G mutant p53) can attenuate the expression of classical wild type p53 responsive target genes, including PTEN, p21, and gadd45 [24]. p53 was found to activate glycogen synthase kinase-3β (GSK-3β) by directly binding to this protein [27]. PTEN phosphorylation at Ser380, Thr382, or Thr383 reduces the activity of PTEN [23]. Moreover, CK2 was found to be the major kinase responsible for the phosphorylation of PTEN at these sites, and GSK-3β was also found to cooperate with CK2 in the phosphorylation of PTEN [1]. The lipid phosphatase PTEN acts on PIP3 to antagonize PI3K signaling pathway and regulates the growth of glioma [6]. GSK-3β mediates the expression of key regulators of the Wnt signal transduction pathway, such as β-catenin, which is the best characterized transcriptional activator in the pathway [7,13]. The activity of GSK-3β is inhibited by phosphorylation of on a serine in the N-terminal domain, Ser9 [12]. However, the involvement of the GSK-3β/PTEN signaling pathway in p53 mutation-regulated tumor cell proliferation is unknown.
We investigated R280T mutation of p53 gene in human glioma SWO-38 cell line. Our results provide direct evidence for the role of mutant p53 in the development of human malignant glioma.

2. Materials and methods

2.1. Reagents

RPMI-1640 medium and Lipofectamine 2000 were purchased from Invitrogen, USA. Anti-phospho-PTEN (p-PTEN, Ser380/Thr382/Thr383), anti-PTEN, anti-p-GSK-3β (Ser9), anti-phospho-Akt (Ser473) and anti-β-catenin antibodies were obtained from Cell Signaling Technology, USA. Anti-human p53 and anti-GAPDH antibodies were products of Bioworld and Chemicon (USA). HRP-conjugated anti-mouse or anti-rabbit IgG secondary antibodies were provided by Jackson ImmunoResearch, USA. Recombinant adenovirus containing cDNA constructs encoding wild-type PTEN (Ad-PTEN) or the empty virus vector were prepared by Beijing SinoGenoMax Co., Ltd., China.

2.2. Cell culture and virus infection

The human glioma cell line SWO-38, derived from a 12-year-old male patient with fibrillary astrocytoma (WHO grade II), was established by Department of Pathology, School of Medicine, Jinan University [18]. Human breast cancer MCF7 cells were provided by Shanghai Institute of Cell Biology, the Chinese Academy of Sciences, China. Cells were maintained in RPMI 1640 culture medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin at 37 °C with an atmosphere of 5% CO2. To induce PTEN overexpression, cells were incubated with 100 multiplicity of infection (MOI) Ad-PTEN recombinant adenovirus or virus vector.

2.3. Polymerase chain reaction (PCR)

Whole genomic DNA for mutation analysis was isolated from SWO-38 cells and was purified using the HiBind DNA column (Omega Bio-Tek, Inc., USA). Oligonucleotide primers were designed to amplify exons 5, 6, and 8 of p53 gene. Specific primers for exons 5, 6 and 8 were designed (Supplementary Table 1). GAPDH served as internal control. PCR profile was as follows: duplication 94 °C, 2 min; 30 cycles of denaturation 94 °C, 30 s, annealing 45 s (Supplementary Table 1) and extension 72 °C for 1 min. The products were examined by sequencing conducted in Life Technologies Corporation, Shanghai, China.

2.4. Immunocytochemical analysis

After fixation, samples were immersed in 3% hydrogen peroxide and incubated with anti-p53 (1:100, Bioword, USA) overnight at 4 °C, followed by incubation with secondary antibody using UltraSensitive™ S–P kit (Maxim, China).

2.5. RNA interference

The siRNA duplex targeting p53 and PTEN were synthesized by RiboBio, Co., Ltd. (Guangzhou, China) as following: p53, 5′-AACATTATTCGACCTTATGGA-3′; PTEN, 5′-GGGATT-3’. A non-silencing sequence was used as negative control. siRNAs were transfected into cells, using lipofectamine™ 2000 [28].

2.6. Flow cytometry analysis

After fixation, cells were incubated at room temperature for 30 min in PBS with 50 mg/ml propidium iodide solution and 50 mg/ml RNase A. Flow cytometry analysis was performed with a FACScan flow cytometer (Becton Dickinson, USA) and data were analyzed by Multicycle software.

2.7. Western blotting

Western blotting was performed as previously described [29]. Whole cell lysates were prepared and electrophoresed on denaturing sodium dodecyl sulfate-polyacrylamide gel and transferred to NC membranes (0.45 μm pore size, PALL, USA). Blots were blocked and probed with primary antibodies for PTEN (1:2000 dilution), p-PTEN (1:100 dilution), p-Akt (1:100 dilution), p53 (1:500 dilution), p-GSK-3βSer9 (1:1000 dilution), β-catenin (1:1000 dilution) and GAPDH (1:4000 dilution). Membranes were then probed with HRP-conjugated secondary antibodies. Immunoreactive bands were visualized by the enhanced chemiluminescence (ECL) assay (Pierce, USA).

2.8. Determination of cell viability

Cells were seeded onto 96-well plate at a density of 1 × 103 cells/well. Cells after treatment were incubated with WST-8 reagent (Dojindo Labs, Japan) at 37 °C for 2 h. Cell viability was determined, according to the absorbance (OD450 nm) measured through a microplate reader (BioRad, USA).

2.9. Statistical analysis

The statistical analysis was performed using the software package SPSS 13.0. Statistical significance was determined using Student’s t-test. Data were presented as mean ± SD. P < 0.05 was recognized as significant different.

3. Results

3.1. Mutation detection of p53 in SWO-38 cells

Genomic DNA obtained from SWO-38 cells was amplified and sequenced for mutations of exons 5, 6, and 8 of p53 gene. The amplified DNA fragments were electrophoresed and the 248, 181, and 231 bp, which matched exons 5, 6, and 8 of p53 gene respectively, were detected (Fig. 1A). The DNA from MCF7 cells was also amplified as a wild-type p53 control for sequencing. The alignment analysis of DNA sequences was performed using NCBI BLAST. A p53 mutation found in SWO-38 cells was G to C transition at codon 280 of p53 exon 8 (AGA coding for arginine — ACA coding for threonine) and it was named R280T (Fig. 1B). However, the amplified DNA sequences of p53 exons 5 and 6 from SWO-38 cells were exactly the same as the human wild type p53 sequences, compared with the wt-p53 sequences from either the amplified MCF-7 genomic DNA or the database. Moreover, an accumulation of p53 protein was observed in the nucleus of SWO-38 cells (Fig. 1C), imply the overexpression of R280T mutant p53 in cells.

3.2. Abrogation of the expression of R280T mutant p53 suppressed the proliferation of glioma cells

RNAi experiments were performed to abrogate the expression of R280T mutant p53 in SWO-38 cells. The mutant p53 protein encoded by R280T mutant p53 was dramatically decreased after transfection of siRNA-p53 (Fig. 2A). Cell proliferation potential was
significantly reduced after silencing of R280T mutant p53, compared with the siRNA-scrambled transfected cells (Fig. 2B). These observations indicated that down-regulation of R280T mutant p53 inhibited the proliferation of glioma cells. Nonetheless, we could not exclude the possibility that decreased number of cells was partially due to the increased cell death. Future study will be continued to explore this possibility.

3.3. Depletion of R280T mutant p53 reduced PTEN phosphorylation and GSK-3β activity

We investigated the involvement of GSK-3β and PTEN in the R280T mutation-mediated cell proliferation. The phosphorylation of GSK-3β at Ser9 was barely detectable under normal conditions in SWO-38 cells, while its level was increased in mutant p53-depleted SWO-38 cells (P<0.05 compared with blank control, Fig. 3A and B), suggesting the down-regulation of R280T mutant p53 may decrease the activity of GSK-3β. A relatively high level of p-PTEN at the site of Ser380, Thr382, or Thr383 was observed in parental SWO-38 cells. However, p-PTEN was significantly decreased after depletion of the mutant p53 in SWO-38 cells (P<0.05 compared with blank control, Fig. 3A and B), suggesting the down-regulation of R280T mutant p53 may promote the activity of PTEN. The off-target effects of RNAi were excluded by comparing the expression of p-PTEN and p-GSK-3βSer9 in the scrambled RNAi transfected cells. These observations indicated that both GSK-3β and PTEN were associated with mutant R280T activity in glioma. Knockdown of the mutant p53 in SWO-38 cells did not impact the β-catenin expression (P>0.05 compared with blank control, Fig. 3A and B). It has been widely accepted that PTEN regulates cell proliferation through PI3K/Akt pathway. Our results revealed that silencing of p53 significantly decreased the level of Akt phosphorylation (P<0.05 compared with blank control, Fig. 3A and B), suggesting PI3K/Akt but not Wnt/β-catenin signaling pathway might be involved in regulating cell proliferation.
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Fig. 3. Western blotting analysis. (A) Silencing the p53 R280T mutation increased p-GSK-3β levels and reduced the PTEN and Akt phosphorylation. Western blotting was performed 48 h after RNAi transfection using either scrambled control or p53 specific siRNAs. Untransfected SWO-38 cells were used as mock control (Blank). (B) Data were quantified from three independent experiments. Fold changes (target protein/GAPDH) were calculated. *P<0.05 compared with scrambled control. (C) Inhibition of GSK-3β activity reduced the phosphorylation of PTEN. SWO-38 cells were treated with LiCl at dose of 0, 1, or 5 mM for 24 h. (D) Data were quantified from three independent experiments. Fold changes (target protein/GAPDH) were calculated. *P<0.05 compared with 0 mM LiCl treatment.

3.4. Inhibition of GSK-3β activity reduced PTEN phosphorylation

To investigate the association between PTEN and GSK-3β in glioma cells, SWO-38 cells were treated with GSK-3β inhibitor LiCl [5]. The p-GSK-3βSer9 level in LiCl treated-cells was significantly increased in a dose dependent pattern (Fig. 3C and D, P<0.05 compared with 0 mM). Whereas, the level of p-PTEN was slightly decreased after 1 mM LiCl treatment but significantly reduced after 5 mM LiCl treatment (Fig. 3C and D, 5 mM, P<0.05 compared with 0 mM), suggesting that PTEN may serve as a down-stream effector of GSK-3β activity.

3.5. Knockdown of PTEN rescued R280T depletion-mediated growth inhibition

To investigate the role of PTEN in glioma cell proliferation, proliferation potential was examined in PTEN-depleted or overexpressing SWO-38 cells. The growth of SWO-38 cells was not impacted by PTEN depletion or overexpression (Fig. 4A, B, E and F). Flow cytometry analysis (Fig. 4C, D, G–I) confirmed that the percentage of the cell population in G0/G1, or G2/M phase was not significantly influenced by RNAi or PTEN adenovirus infection, suggesting that impairment of PTEN function occurred in SWO-38 cells due to inactivation after phosphorylation. However, knockdown of both PTEN and R280T could significantly rescue the growth inhibition mediated by the depletion of R280T mutant p53 (Fig. 4J), suggesting that R280T might promote glioma cell growth through inhibition of PTEN.

4. Discussion

Tumor-derived p53 mutations are found in approximately half of all human cancers [15,25,26], and mutant p53 is closely associated with the hallmarks of cancer [19]. p53 mutations abrogate its cardinal functions in promoting apoptosis, cell-cycle arrest, and DNA repair, thereby leading to cancer progression [25,26]. The point mutations of p53 in human malignant gliomas were found clustered in several hot-spots located in its exons 4-8 [8]. Sun et al. reported a heterozygous G→C mutation at codon 280(exon 8) (also known as R280T), which causes an Arg→Thr substitution of the p53 gene in the human nasopharyngeal carcinoma (NPC) cell line [20]. In the TP53 mutation database established by IARC, the mutation of R280T was found in a few tumor cell lines, including cell lines of NPC [20], bladder carcinoma [4,16], breast carcinoma [3], gastric and esophageal adenocarcinoma [14]. Nevertheless, this mutation has not yet been identified in human malignant glioma cell lines. Here, we examined the potential hot-spot mutations in exons 5, 6, and 8 of the p53 gene in human SWO-38 glioma cells and only found the R280T mutation in exon 8.

The tumorigenic effects of the R280T mutation in p53 varied in different types of cells. Ectopic expression of R280T mutant p53 in nontumorigenic human Saos-2 cells that lack endogenous p53 promote anchorage-independent growth and in vivo tumorigenicity [21]. However, forced expression of the R280T mutant p53 in mouse nontumorigenic JB6 variants that bear endogenous wild-type p53 enhanced the anchorage-independent growth in vitro but failed to form tumor in nude mice [21]. Moreover, silencing the R280T mutation in CNE-2 nasopharyngeal carcinoma cells increased the
expression of p21 [24]. We found that abrogating the expression of R280T mutant p53 using a specific RNAi significantly inhibited cell proliferation in SWO-38 cells, suggesting that the R280T mutation may lead to gain-of-function activities in human malignant gliomas.

PTEN is an endogenous inhibitor of the PI3K/Akt signaling pathway. PTEN mutations occur after p53 mutations in glioma development [10]. GSK-3β is a multi-functional kinase and its kinase activity is regulated by site-specific phosphorylation. Complete activation of GSK-3β generally requires phosphorylation at Tyr216, whereas phosphorylation at Ser21 inhibits GSK-3β activity [7]. GSK-3β phosphorylates several components of the Wnt signaling pathway, including β-catenin [7,13]. We investigated signaling pathways that might be involved in p53 mutation-regulated tumor cell proliferation. Our results indicated that the GSK-3β/PTEN signal pathway may be associated with the R280T mutation-mediated growth of SWO-38 cells, because depletion of R280T mutant p53 led to induction of p-GSK-3β but to a reduction of p-PTEN. Furthermore, inhibition of GSK-3β activity by LiCl attenuated PTEN phosphorylation at Ser380/Thr382/Thr383 reduces PTEN activity [23]. Although CK2 was found to be the major kinase responsible for the phosphorylation of PTEN at Ser380/Thr382/Thr383, GSK-3β was also found to cooperate with CK2 in the phosphorylation of PTEN [1]. Therefore, it seems that GSK-3β may also negatively regulate PTEN activity by inducing the phosphorylation of Ser380/Thr382/Thr383. However, our results excluded the involvement of the Wnt/β-catenin signaling pathway. Furthermore, knockdown of PTEN had no direct effects on cell proliferation or cell cycle in SWO-38 parental cells, but rescued R280T depletion-mediated growth inhibition.

Collectively, we show that the p53 gene mutation at codon 280 of exon 8 regulates proliferation of human glioma cells through GSK-3β/PTEN signal pathway. Further studies will be needed to confirm these results in other mutant p53 expressing cells or mutant p53 overexpressing cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2012.09.022.

References


