Effects of INPP4B gene transfection combined with PARP inhibitor on castration therapy—Resistant prostate cancer cell line, PC3

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Received 9 September 2013; received in revised form 26 November 2013; accepted 26 November 2013

Abstract

Objective: This study investigated the effects of combining inositol polyphosphate-4-phosphatase type II (INPP4B) gene transfection with poly(adenosine diphosphate—ribose) polymerase (PARP) inhibitor on castration therapy—resistant prostate cancer cell line, PC3.

Materials and methods: PC3 and LNCap cells were cultured in vitro, and the expression of INPP4B at the messenger RNA level was detected using reverse transcription polymerase chain reaction. PC3 cells transfected with recombinant lentivirus vector carrying the human INPP4B gene, and the expression of INPP4B was confirmed using real-time polymerase chain reaction and Western blot. The effect of INPP4B transfection or PARP inhibitor treatment or both on the proliferation, apoptosis and cell cycle, and level of protein kinase B (p-AKT) of PC3 cells was determined using Cell Counting Kit-8 assay, flow cytometry, and Western blot, respectively.

Results: INPP4B gene was lost in PC3 cells and successfully expressed in PC3 cells after recombinant lentivirus transfection. Cell proliferation was inhibited and the level of p-AKT decreased, causing a G1 arrest. PARP inhibitor had a remarkable negative effect on cell proliferation and promoted apoptosis, conferring a G2/M arrest, which was dose dependent; however, the level of intracellular p-AKT showed a slight rise. INPP4B gene transfection and PARP inhibitor combined blocked cell cycle progression in G1 phase, enhanced the inhibition of cell proliferation, and kept low intracellular p-AKT level.

Conclusions: Our results demonstrated that the combination of INPP4B gene transfection and PARP inhibitor had a synergistic antitumor effect on PC3 cells, which was expected to shed new light on combined biological therapy in castration therapy—resistant prostate cancer.

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Keywords: INPP4B; Combined; PARP inhibitor; Castration therapy—resistant prostate cancer; PC3; PTEN

1. Introduction

Prostate cancer is one of the most commonly diagnosed malignant tumors in men, also the second leading cause of cancer-related deaths [1]. Cases that fail to benefit from androgen deprivation therapy [2], known as castration therapy—resistant prostate cancer [3], mainly depends on chemotherapy, whose effects are still poor. Understanding the pathogenesis and exploring new targets of castration therapy—resistant prostate cancer may potentially provide significant clinical effect [4].

Polyphosphate-4-phosphatase type II (INPP4B) dephosphorylates phosphatidylinositol-3,4-bisphosphate, whose levels determine protein kinase B (p-AKT) activity, plays a crucial role in negative regulation of phosphatidylinositol 3-kinase (PI3K) pathway, and blocks the cell malignant transformation [5,6]. At present, it is known that INPP4B is involved in a wide variety of tumors and emerges as a potential tumor suppressor [7,8]. Androgen receptor induces expression of INPP4B at the level of transcription in human prostate cancer cell lines is the only known regulatory mechanism so far [6], and Miyamoto et al. [9] speculated that castration therapy—resistant prostate cancer cells may have lost androgen receptor expression. Hodgson et al. [6] had found a significant decrease in INPP4B expression in human prostate cancers compared with benign prostate...
epithelium tissue by examination of radical prostatectomy specimens. All of these led to the hypothesis that INPP4B may exert lower or no expression in castration therapy-resistant prostate cancer.

The poly(adenosine diphosphate–ribose) polymerase (PARP) family is mainly located in the nucleus, and it participates in repairing both single- and double-stranded DNA breaks by mechanism of base excision repair [10]. Function loss of phosphatase and tensin homolog is frequently observed in prostate cancer [11], contributing to defective homologous recombination [12]. PARP inhibitor can promote cell death by inhibiting the compensatory DNA repair pathway in phosphatase and tensin homolog-null tumor [13,14]. However, evidence suggested that inhibition of PARP resulted in activation of the PI3K/AKT pathway [15,16], which may counteract the antitumor effect and associate with cytostatic resistance.

We hypothesized that the transfecting INPP4B gene would not only have antiproliferative effects on castration therapy–resistant prostate cancer cell line, PC3, but also reduce the level of p-AKT. At the meantime, we want to know whether it would have a synergistic antitumor effect when combined with PARP inhibitor.

2. Materials and methods

2.1. Cell culture

PC3 [17] and LNCaP cells (as a positive control, who were proved expression INPP4B [6]) were preserved by Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Shandong University (Jinan, China) and were maintained in Roswell Park Memorial Institute (RPMI) 1640 media and supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin. RPMI 1640 media and fetal bovine serum were purchased from Gibco (New York, US); penicillin-streptomycin was purchased from Beyotime Institute of Biotechnology (China). Cells were cultured at 37°C in a humidified environment in the presence of 5% CO2.

2.2. Transfection

PC3 cells were transfected with recombinant lentivirus vector carrying the human INPP4B (Lenti-INPP4B, previous DNA sequencing analysis revealed successful construction and its titer was 2 × 108 TU/ml), using polybrene as recommended (GeneChem Co, Shanghai, China). Briefly, exponentially proliferating cells were seeded into 24-well culture plates and incubated overnight, allowing cells to adhere. Multiplicity of Infection (MOI) = 40 was adopted. To each well, 40 μl of Lenti-INPP4B or 8 μl of Lenti-Green Fluorescent Protein (GFP) (negative control, reflects the transfection efficiency of Lenti-INPP4B indirectly and its titer was 1 × 106 TU/ml) was added. After 5 days of transfection, cells were collected; RNA and protein were extracted to analyze the expression of INPP4B in PC3 cells; cells were seeded into well plates; and CCK-8 and flow cytometry were used to assess cell vitality, apoptosis, and cell cycle. All experiments were run with at least 3 replicate cultures and repeated 3 times.

2.3. Polymerase chain reaction analysis

RNA was prepared from cell line using TRIzol reagent as described by the manufacturer (CoWin Biotech Co, Beijing, China). First strand complimentary DNA was synthesized using the reverse transcription kits, INPP4B (forward: GAGGATCCCCGGGTACCGTCGCCACCATGGAATT-TAAAGAGGAAAGGGCATCAG, reverse: TCATCTTGTA-

TAGTCGTGGTGCAGCTTTTCCATAATGCTCCCTCTTG), GAPDH (forward: GCACCGTCAAGGCTGAGAAC, reverse: TGGTGAAGAGCGCCAGTGGA). The amplification system of complimentary DNA was followed by the manufacturer. Reverse transcription polymerase chain reaction (PCR): equal amounts of each reaction product’s DNA were run on 2% agarose gel, and the intensity of the bands in the gels was sequentially visualized. Real-time quantitative PCR: reaction conditions of Ultra SYBR Green quantitative PCR with X-Rhodamine (ROX) reaction system were followed by the manufacturer. To calculate the relative content of INPP4B messenger RNA (mRNA), 2−ΔΔCt was adopted.

2.4. Western blot analysis

We set up control by untreated PC3 cells, Lenti-GFP, transfection INPP4B gene (Lenti-INPP4B), PARP inhibitor, AG014699 (Selleck Chemicals, Texas, USA), Lenti-GFP combined with PARP inhibitor, AG014699 (Lenti-GFP + PARP inhibitor), and Lenti-INPP4B combined with PARP inhibitor, AG014699 (Lenti-INPP4B + PARP inhibitor) 6 groups (same grouping method for cell viability test and flow cytometry). PARP inhibitor, AG014699, was diluted with 0.1% dimethyl sulfoxide to a 1 mmol/l(mM) stock solution. Cells (7 × 105 cells/well) were seeded into 6-well plate. After 48 hours, cells were lysed in radioimmunoprecipitation assay buffer (supplemented with protease inhibitors, phenylmethanesulfonyl fluoride, phenylmethanesulfonyl fluoride-radioimmunoprecipitation assay = 1:100) and phosphatase inhibitor cocktail. Protein density was measured using bicinchoninic acid assay. One hundred μg (per lane) of protein lysate was separated using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Millipore, Massachusetts, USA). The membranes were blocked in bovine serum albumin and probed with the following primary antibodies: anti-INPP4B (1:500), anti-p-AKT Ser473 (1:500), and anti-AKT (1:500). They were blocked in bovine serum albumin and probed with the following primary antibodies: anti-INPP4B (1:500), anti-p-AKT Ser473 (1:500), and anti-AKT (1:500). They were blocked in bovine serum albumin and probed with the following primary antibodies: anti-INPP4B (1:500), anti-p-AKT Ser473 (1:500), and anti-AKT (1:500). They were blocked in bovine serum albumin and probed with the following primary antibodies: anti-INPP4B (1:500), anti-p-AKT Ser473 (1:500), and anti-AKT (1:500). They were blocked in bovine serum albumin and probed with the following primary antibodies: anti-INPP4B (1:500), anti-p-AKT Ser473 (1:500), and anti-AKT (1:500). They were blocked in bovine serum albumin and probed with the following primary antibodies: anti-INPP4B (1:500), anti-p-AKT Ser473 (1:500), and anti-AKT (1:500). They were blocked in bovine serum albumin and probed with the following primary antibodies: anti-INPP4B (1:500), anti-p-AKT Ser473 (1:500), and anti-AKT (1:500). They were blocked in bovine serum albumin and probed with the following primary antibodies: anti-INPP4B (1:500), anti-p-AKT Ser473 (1:500), and anti-AKT (1:500). They were blocked in bovine serum albumin and probed with the following primary antibodies: anti-INPP4B (1:500), anti-p-AKT Ser473 (1:500), and anti-AKT (1:500). They were blocked in bovine serum albumin and probed with the following primary antibodies: anti-INPP4B (1:500), anti-p-AKT Ser473 (1:500), and anti-AKT (1:500). They were blocked in bovine serum albumin and probed with the following primary antibodies: anti-INPP4B (1:500), anti-p-AKT Ser473 (1:500), and anti-AKT (1:500). They were blocked in bovine serum albumin and probed with the following primary antibodies: anti-INPP4B (1:500), anti-p-AKT Ser473 (1:500), and anti-AKT (1:500). They were blocked in bovine serum albumin and probed with the following primary antibodies: anti-INPP4B (1:500), anti-p-AKT Ser473 (1:500), and anti-AKT (1:500). They were blocked in bovine serum albumin and probed with the following primary antibodies: anti-INPP4B (1:500), anti-p-AKT Ser473 (1:500), and anti-AKT (1:500). They were blocked in bovine serum albumin and probed with the following primary antibodies: anti-INPP4B (1:500), anti-p-AKT Ser473 (1:500), and anti-AKT (1:500). They were blocked in bovine serum albumin and probed with the following primary antibodies: anti-INPP4B (1:500), anti-p-AKT Ser473 (1:500), and anti-AKT (1:500). They were blocked in bovine serum albumin and probed with the following primary antibodies: anti-INPP4B (1:500), anti-p-AKT Ser473 (1:500), and anti-AKT (1:500).
primary antibodies were purchased from Cell Signaling (Massachusetts, USA).

2.5. Cell Counting Kit-8 analysis

The measurement of viable cell mass was performed with Cell Counting Kit-8 (CCK-8) (Beyotime Institute of Biotechnology, China). Cells (7 \times 10^5 cells/well) were first seeded into 96-well flat-bottomed plates for 8 hours to adhere. Treatment with 0.1, 1, 10, 20, and 40 \mu M of the PARP inhibitor, AG014699, for 48 hours as single agent was administered to determine the concentration of the drug that was required to achieve 50% inhibitory concentration (IC50), then IC50 was selected as the fixed single concentration in combination treatments. After 24, 36, and 48 hours, 10 \mu l of solution from Cell Counting Kit-8 was added to each well. These plates were continuously incubated for 45 minutes in a humidified CO2 incubator at 37°C. Finally, the absorbance of sample taken from each well was measured at 450 nm, on the basis of which the percentage of surviving cells of each treated group to the untreated one was plotted.

2.6. Flow cytometry analysis

Detection of apoptosis and cell cycle was done using flow cytometry. The cells were harvested after 48 hours. Apoptosis assay: cells were washed with PBS and processed for labeling with annexin V/propidium iodide as described by the manufacturer (Beyotime Institute of Biotechnology, China). Cell cycle assay: cells were fixed in 70% precooling ethanol for 24 hours, processed for labeling with propidium iodide, and kept in dark for 30 minutes. For each sample, 10,000 cells were acquired; cell populations under G0/G1, S, and G2/M phases were determined by employing PI fluorescence regions with FL2 area vs. FL2 width. Analysis was completed using Gallios Cytometry list mode data acquisition and analysis software on a Gallios Flow Cytometer (Beckman Coulter, Kraemer Boulevard Brea, CA, USA).

2.7. Statistical analysis

SPSS 17.0 statistical software was used for statistical analysis. Statistical comparisons of mean values were calculated using the 2-sided Student t test with the assumption of unequal variances. \( P < 0.05 \) was considered significant. All data were presented as mean \( \pm \) standard deviation.

3. Results

3.1. INPP4B was not detected in PC3 cells at the level of mRNA

Initially, we evaluated the expression of INPP4B at the mRNA level in PC3 cells. Result showed that INPP4B gene was not detected in PC3 cells (Fig. 1).

3.2. INPP4B was successfully expressed in PC3 cells

To investigate the role of INPP4B on cell vitality in human castration therapy–resistant prostate cancer cell line, PC3, INPP4B gene was transfected into PC3 cells. To confirm the expression of INPP4B at the mRNA and protein level, real time PCR and Western blot assay were performed. The relative levels of INPP4B mRNA in Lenti-INPP4B group and Lenti-GFP group were 87.84 \( \pm \) 7.62 and 1.08 \( \pm \) 0.03, respectively (\( P < 0.05 \)) (Fig. 2A). Similar results were obtained using Western blot analysis. Noticeable expression of INPP4B protein was shown in the Lenti-INPP4B group, with no INPP4B expression in Lenti-GFP group (Fig. 2B).
3.3. Effect of transfecting INPP4B gene on proliferation, cell cycle, and apoptosis of PC3 cells

We were interested in validating the observation that PC3 cells were sensitive to transfecting INPP4B gene. Following continuous treatment with Lenti-INPP4B alone for 24, 36, and 48 hours, cell vitality was determined using the CCK-8 Kit. Compared with control, the vitality of cells was 86.91% ± 7.70%, 64.27% ± 3.12%, and 47.57% ± 7.74% in the Lenti-GFP group and 66.18% ± 2.32%, 45.10% ± 0.00%, and 29.35% ± 3.19% in Lenti-INPP4B group after 24, 36, and 48 hours, respectively. It demonstrated transfecting INPP4B gene resulted in a significant reduction of cell growth (P < 0.05) (Fig. 3).

Transfecting INPP4B gene into PC3 cells induced an...
accumulation of G1 phase, and the rate of G1 phase was 64.25% ± 3.99%, 65.32% ± 2.59%, and 76.64% ± 1.97% in control by untreated PC3 cells, Lenti-GFP group and Lenti-INPP4B group, respectively (Fig. 5B). Apoptosis rate measured using flow cytometry did not increase significantly in the Lenti-INPP4B group compared with the Lenti-GFP group (Fig. 5C).

3.4. Effect of PARP inhibitor, AG014699, on proliferation, cell cycle, and apoptosis of PC3 cells

AG014699 showed significant antiproliferative effects in a dose-dependent pattern. Cells vitality was 93.58% ± 3.51%, 64.63% ± 2.18%, 57.15% ± 3.38%, 43.88% ± 3.02%, and 40.60% ± 0.86%, 48 hours after treatment with 0.1, 1, 10, 20, 40 μM of AG014699 and dimethyl sulfoxide, respectively (Fig. 4A). As 0.1, 1, and 10 μM were less than IC_{50}, 10 μM was selected as the fixed single concentration for use and in combination treatments. Next, we were interested in whether AG014699 decreased the proliferation rate of PC3 cells in a time-dependent pattern. We detected the cell vitality after treatment with 10-μM AG014699 at different times. The results were positive, the vitality of PC3 cells was 88.53% ± 1.73%, 60.77% ± 2.26%, and 54.12% ± 1.96% at 24, 36 and 48 hours, respectively (Fig. 4B).

Fig. 5. Antitumor effects of transfecting of INPP4B gene combined with PARP inhibitor treatment on PC3 cells. (A) The change of cell number and shape under the microscope. (B) The viability of PC3 cells measured using CCK-8. (C) The cell cycle phase distribution of PC3 detected by flow cytometry. (D) Apoptosis of PC3 cells detected using annexin V-FITC/PI staining. (Asterisks denote statistical significance between Lenti-INPP4B + PARP inhibitor and Lenti-INPP4B and PARP inhibitor treatment, *P < 0.05). (Color version of figure is available online.)
showed the ratio of G2/M phase were 23.50% ± 2.90%, 35.10% ± 5.11% and 55.97% ± 3.75% when treated with 1, 10, and 20 μM after 48 hours, which implied that the ratio of G2/M phase increased with the mounting concentration of AG014699. A high concentration of PARP inhibitor can induce a G2/M accumulation (Fig. 4C). Apoptosis assay was analyzed using annexin V-FITC/PI staining: AG014699 could promote cell apoptosis in a dose-dependent pattern (Fig. 4D).

3.5. Effect of transfecting of INPP4B gene combined with PARP inhibitor treatment on proliferation, cell cycle, and apoptosis of PC3 cells

The aforementioned results revealed that PC3 cells were sensitive to both INPP4B transfection and PARP inhibitor treatment. Now we wonder what would happen if the treatments were combined. First, observed under the microscope, we found the number of PC3 cells decreased significantly and the shape of PC3 cells became irregular after treatment with transfecting INPP4B gene combined with PARP inhibitor treatment compared with single treatment (Fig. 5A). Next, we tested the proliferation of PC3 cells under the combined treatment. Compared with control, the vitality of cells was 49.85% ± 0.30%, 26.30% ± 1.81%, and 9.72% ± 3.10% after 24, 36, and 48 hours. Significant growth inhibition could be observed upon combination than transfection of INPP4B gene and PARP inhibitor given separately (P < 0.05) (Fig. 5B). Then the cell cycle distribution was detected. Transfection of INPP4B gene combined with PARP inhibitor treatment caused a G1 arrest (Fig. 5C). Finally, we analyzed cell apoptosis, finding that compared with PARP inhibitor alone, combination with transfection of INPP4B gene did not result in any further significant increase apoptosis rate (Fig. 5D).

3.6. Effect of transfecting of INPP4B gene combined with PARP inhibitor treatment on the levels of p-AKT in PC3 cells

To determine why transfection of INPP4B gene combined with PARP inhibitor treatment had a synergistic antitumor effect on PC3 cells, we decided to detect the change of p-AKT level. Western blot analysis was performed to determine the levels of p-AKT following 48 hours of treatment. The levels of p-AKT decreased upon treatment with transfection of INPP4B gene, but not in Lenti-GFP (Fig. 6A). The levels of p-AKT increased upon treatment with PARP inhibitor, AG014699, in a dose-dependent manner (Fig. 6B). Compared with AG014699 used alone, the levels of p-AKT decreased when combined with transfection of INPP4B gene, which was not detected when combined with Lenti-GFP (Fig. 6A). It implied transfection INPP4B gene could antagonize the elevated p-AKT levels caused by AG014699 in PC3 cells.

4. Discussion

Loss of heterozygosity of INPP4B is frequently observed in a wide variety of tumors, e.g., hormone receptor-negative breast cancer and ovarian cancer, associated with the magnitude Akt activation [18–20]. Our previous laboratory data showed that INPP4B had a lower expression in triple-negative breast cancer cell line, MDA-MB-231. Similarly, INPP4B gene was lost in castration therapy-resistant prostate cancer, PC3 cells. We found that after PC3 cells was transfected with INPP4B gene, the viability of cells decreased significantly accompanied by reduction of the p-AKT levels, which showed concordance with the results of Fedele [7], causing a G1 arrest. This indicated that reexpression of INPP4B in INPP4B gene-negative cancers could inhibit tumor growth.

PARP inhibitors might kill tumor cells by inactivating the single-strand break repair pathway [21]. Our experiment showed that AG014699 had significant antiproliferative effects in both a time- and a dose-dependent manner. Moreover, as the concentration increased, necrosis or apoptosis occurred and a G2/M accumulation on cell cycle progression was induced. Szanto et al. [16] have suggested that PARP inhibitors induced activation of the PI3K/AKT pathway. Our study further proved the hypothesis by
showing that the level of p-AKT was increased by PARP inhibition of AG014699 in a dose-dependent pattern.

As most targeted therapies used as single agents frequently increase the risk of resistance, current efforts toward treatment enhancement focus on identifying optimal ways of targeting combinations of specific molecular defects present in each cancer [22–25]. The study by Kimbung et al. [25] showed that cotargeting of the PI3K pathway improved the response of BRCA1-deficient breast cancer cells to PARP inhibition. Thus in our experiment, we design to combine the transfection of INPP4B gene with PARP inhibitors in PC3 cells. The results showed a synergistic effect on inhibiting proliferation of PC3 cells. Nevertheless, compared with PARP inhibitor alone, the proportion of cell apoptosis did not result in any further significant increase when they were combined. The combining intervention would block cells in G1. It was interesting that the transfection of INPP4B gene could antagonize the elevated p-AKT level caused by AG014699 in PC3 cells.

Conclusion

Our study is unique in investigating and reporting that INPP4B gene transfection combined with PARP inhibitor has a synergistic antitumor effect on castration therapy–resistant prostate cancer cell line, PC3, which is expected to become the optimal choice of combined biological therapy in castration therapy–resistant prostate cancer.

Acknowledgments

This work was supported by Grants from the Shandong province science and technology development projects (2013GSF11839).

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