Tumor Biol.
DOI 10.1007/s13277-013-1589-y

RESEARCH ARTICLE

INPP4B overexpression enhances the antitumor efficacy of PARP inhibitor AG014699 in MDA-MB-231 triple-negative breast cancer cells

Ying Sun · Huan Ding · Xinguang Liu · Xiaoqing Li · Li Li

Received: 3 November 2013 / Accepted: 19 December 2013

Abstract Although preclinical and clinical studies on poly-(adenosine diphosphate ribose) polymerase (PARP) inhibitor alone or in combination with DNA-damaging agents have shown promising results, further research to improve and broaden the application scope of this therapeutic approach is needed. The main aim of this study was to evaluate whether overexpressing inositol polyphosphate 4-phosphatase type II (INPP4B) gene, a novel tumor suppressor gene negatively regulating the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway, could enhance the antitumor efficacy of PARP inhibitor AG014699 used in the treatment of triple-negative breast cancer (TNBC). Here in this report, we used a TNBC cell line MDA-MB-231 without expression of INPP4B as the study model and a lentiviral system to stably overexpress INPP4B gene in MDA-MB-231 cells. We detected that the overexpression of INPP4B could significantly suppress cell proliferation and block cell cycle progression in G1 phase via decreasing the protein level of phosphorylated AKT. It is further revealed that PARP inhibitor AG014699 induced DNA damage conferring a G2/M arrest and decreased cell viability, which is paralleled by the induction of apoptosis. However, PARP inhibitor AG014699 could activate the PI3K/AKT signaling pathway activity and partially offset its therapeutic efficacy. In our study, a significant enhancement of proliferation inhibition was observed when INPP4B overexpression was combined with PARP inhibitor AG014699 in comparison with either single treatment. The suppression of PI3K/AKT pathway caused by the overexpression of INPP4B contributed to the enhanced antitumor efficacy of the combined therapy. Our in vitro results indicated that this experimental therapeutic strategy combining INPP4B overexpression and PARP inhibitor AG014699 might be of potential therapeutic value as a new strategy for the treatment of patients with TNBC and is worthy of further study.

Keywords INPP4B · PARP inhibitor · PI3K/AKT · Antitumor · Triple-negative breast cancer

Introduction

Triple-negative breast cancer (TNBC) is defined immunohistochemically as any breast tumor that lacks the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), accounting for approximately 10–15 % of all breast cancers [1]. Compared with other subtypes, TNBC has a shorter disease-free survival (DFS) and overall survival (OS), with the characteristics of high grade of malignancy, quick progression, strong invasiveness, and high rate of recurrence and metastasis [2, 3]. It has been demonstrated that TNBC patients do not benefit from ER/PR endocrinotherapy or anti-HER2 molecular therapy, and therefore, chemotherapy is left as their only option. In order to improve the clinical outcomes of TNBC patients, it is urgent to look for more effective treatment modalities.

Recently, inositol polyphosphate 4-phosphatase type II (INPP4B), an important lid phosphatase enzyme maintaining homeostasis of intracellular phosphoinositides, has emerged as a putative tumor suppressor gene [4]. INPP4B plays a

Ying Sun and Huan Ding contributed equally to this work.

Y. Sun · H. Ding · L. Li (✉)
Department of Medical Oncology, Cancer Center, Qilu Hospital of Shandong University, No. 107 Wenhuaxi Road, Jinan 250012, China
e-mail: drlili5060@163.com

X. Liu
Department of Hematology, Cancer Center, Qilu Hospital of Shandong University, No. 107 Wenhuaxi Road, Jinan 250012, China

X. Li
School of Medicine, Zhejiang University, Hangzhou 310058, China

Published online: 14 January 2014

crucial role in the negative regulation of the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway and blocks the malignant transformation of cancer [5-7]. Dysregulation of the INPP4B will lead to excessive activation of PI3K/AKT signaling pathway. Tumorigenic advantages driven by inappropriate activation of the PI3K/AKT pathway include increased cell proliferation, migration, angiogenesis, evasion of apoptosis, and genome instability. INPP4B protein expression has also been assessed in normal human mammary tissue sections. In epithelial cells comprising the mammary ducts, INPP4B expression was restricted to luminal epithelial cells and appeared absent in surrounding myoepithelial cells [5]. Loss of heterozygosity (LOH) in INPP4B gene is frequently observed in human breast cancer susceptibility gene (BRCA) mutant and hormone receptor-negative breast cancers, associated with high tumor grade and shortened survival [8-10]. The study by McAuliffe et al. has also suggested that the PI3K pathway was excessively activated in TNBC patients [11].

Cancer cells frequently harbor defects in DNA repair pathways which lead to genomic instability. This can foster tumorigenesis but also provide a weakness in the tumor that can be exploited therapeutically. DNA microarray analyses have shown that about 30 % of patients with TNBC are positive for BRCA mutations [12-14]. BRCA pathway is important for DNA double-strand break repair by homologous recombination (HR) which can restore the original nucleotide sequence and maintain the stability of genome [15, 16]. When the BRCA-associated double-strand repair pathway is impaired or dysfunctional, alternative single-strand base excision repair (BER) which is dependent on poly-(adenosine diphosphate ribose) polymerase (PARP) would take into action [17]. It has been further shown that PARP plays very important roles in the process of repairing DNA breaks by binding to the DNA lesions and recruiting downstream repairing effectors [18]. According to the theory of synthetic lethality [19], in HR-defective breast cancer cells with BRCA mutations, the application of PARP inhibitors might kill tumor cells by inactivating the single-strand break repair pathway and cause subsequent accumulation of irreparable double-strand breaks [20, 21]. Interestingly, although recent research suggested that PARP inhibitors promoted apoptosis by inhibition of DNA repair, they could induce the AKT phosphorylation at the same time. Activation of the PI3K/AKT pathway can partially counteract the antitumor activity of PARP inhibitors, which could result in drug resistance [22, 23].

Like in other tumor cell lines, upon treatment of PARP inhibitors, the protein level of phospho-AKT (p-AKT) might increase in TNBC cell lines harboring BRCA mutation. At the same time, INPP4B overexpression could downregulate p-AKT levels. It is reasonable to speculate that a combining treatment of INPP4B overexpression and PARP inhibitors in BRCA mutant cells should be more effective compared with targeting single molecule alone. In the present study, the antitumor effects of INPP4B overexpression via lentiviral transduction combined with PARP inhibitor AG014699 were tested in BRCA mutant TNBC cell line MDA-MB-231, and the results suggested that the cytotoxic effect of combining INPP4B overexpression and PARP inhibitor was superior to either single treatment. This combination treatment might represent an improved selective targeted therapeutic strategy for breast cancers with concomitant DNA damage repair defects and dysregulated PI3K signaling as well as for sporadic tumors with a "BRCAness" of TNBC phenotype [24].

Materials and methods

Cell culture

Human breast cancer cell lines MDA-MB-231 (ER−/PR−/HER2−) and MCF-7 (ER+/PR+) were obtained from the School of Life Sciences of Shandong University (Jinan, Shandong, China). The human embryonic kidney cells 293 T used for the detection of transfection were provided by Department of Anatomy, Shandong University. Cells were cultured in RPMI-1640 medium supplemented with 2 mmol/L of L-glutamine, 10 % fetal bovine serum (FBS), 100 IU/mL of penicillin, and 100 mg/mL of streptomycin. Cells were cultured at 37 °C in a humidified chamber supplemented with 5 % CO_2. Stock solutions of PARP inhibitor AG014699 provided by Selleck were made in DMSO (≤0.01 %).

Construction of plasmids and transfection of cells

The fragment of INPP4B was acquired from amplification using a polymerase chain reaction (PCR) technique with the upstream primer 5′-GAGGATCCCCGGGTACCGGTCGCCACCATGGAAATTAAAGAGGAAGGGGCATCAG-3′ and downstream primer 5′-TCATCCTTGTAGTCGCTGGTGTCAGCTTTTCCATAAGTCCCCTCTG-3′. After double digestion with endonucleases Nhe I and Age I, target synthetic gene segment was linked to vector PGC-LV to construct plasmid. The negative control group contained a green fluorescent protein (GFP) sequence. Lentiviral vector DNAs and packaging vectors (pHelper1.0, pHelper2.0) (GeneChem) were then transfected into 293 T cells using Lipofectamine™ 2000 Reagent (Invitrogen, USA) according to the manufacturer's instructions. Supernate was harvested at 48 h posttransfection, and viral concentrate was obtained through further centrifugation. MDA-MB-231 cells were grown to 70–80 % confluence and infected with lentivirus into six-well plates, at multiplicity of infection (MOI) of 10 and 20. MDA-MB-231 cells from the Lenti-INPP4B group contained a target synthetic gene segment. For the negative control group Lenti-GFP, MDA-MB-231 cells contained a

green fluorescent protein sequence to determine the efficiency of transfection. The control group MDA-MB-231 cells were just cultured in RPMI-1640 medium.

Detection of the mRNA level expression of INPP4B gene

DNA agarose gel electrophoresis was used to initially detect the expression of INPP4B mRNA in MDA-MB-231 cells, and MCF-7 cells were served as positive controls. RNA was extracted using TRIzol Reagent (Beyotime), and RNA concentration and purity were measured by NanoDrop 1000 (Thermo, USA). Ten nanograms of RNA was taken for cDNA reverse transcription. Actual conditions and steps of reaction shall be in accordance with the specification. INPP4B includes upstream primer 5′-TGACTGGTGTCATTCCCAT-3′ and downstream primer 5′-GCATAGCCCAAGAAACTT CGC-3′; GAPDH includes upstream primer 5′-GCACCGTC AAGGCTGAGAAC-3′ and downstream primer 5′-TGGTGA AGACGCCAGTGGA-3′. Equal amounts of PCR product were run on 2 % agarose gel, and the intensity of the bands in the gels was sequentially visualized.

Real-time PCR

MDA-MB-231 cells were infected with lentivirus into six-well plates, at MOI of 20. On day 5, RNA could be reversely transcribed into cDNA. Ultra SYBR Green qPCR was utilized with ROX to proceed with the real-time quantification (ABI 7900HT). Relative transcript level of mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method.

The reaction conditions of real-time quantification (CWBIO)

Initial denaturation was at 95 °C for 5 s, denaturation at 95 °C for 30 s, annealing at 60 °C for 15 s, and elongation at 72 °C for 45 s, followed by 30 cycles. The solubility curve and amplification curve would be drawn after the reaction was finished.

Cell Counting Kit-8 assay

The impacts on the cell proliferation by INPP4B overexpression, PARP inhibitor AG014699 alone, or in combination were studied. MDA-MB-231-transfected and MDA-MB-231-nontransfected cells were inoculated into 96-well plates with a density of 7×10^3 cells/well. The growth curve was calculated to evaluate the vector-infected cell viability (from days 1 to 5).

Drug treatment

PARP inhibitor AG014699 was applied at five concentration gradients as 0.1, 1, 10, 20, and 40 μM. Cell Counting Kit-8 (CCK-8) method was used to detect the proliferation rate of cell after drug intervention for 48 h. The one close to GI_{50} (50 % growth inhibition concentration) was selected as a treatment concentration alone or combined with INPP4B overexpression in MDA-MB-231 cells. The action time was determined using the trial design.

Specific steps

One hundred-microliter culture medium containing 10 μl CCK-8 was added to each microtiter well and incubated for 1 h at 37 °C to form water dissoluble formazan. The amount of the formazan dye (450 nm absorbance) generated by dehydrogenases in the cells is directly proportional to the number of living cells. Each assay was carried out in triplicate.

Western blotting

Total protein (TP) was extracted by collected MDA-MB-231 cells to detect the expression of INPP4B. MCF-7 cells which have been proved to have high levels of INPP4B expression were used as positive controls. Next, TP was extracted by collected vector-infected MDA-MB-231 cells to determine whether transfection had been successful. MDA-MB-231-transfected and MDA-MB-231-nontransfected cells treated with different concentrations of the PARP inhibitors AG014699 (10, 20, and 40 μM) for 24 h were collected to detect the expression of p-AKT. Cells were lysed in RIPA buffer (CWBIO, China) containing protease inhibitors, and the samples were boiled five times in a loading buffer for 5 min. For protein quantity analysis, equal amounts of proteins (100 μg) were run on a 10 % SDS-PAGE gel and transferred to polyvinylidene fluoride membranes. The membranes were blocked in BSA for 2 h at room temperature followed by overnight incubation with primary rabbit/rat monoclonal antibodies against INPP4B, AKT, p-AKT (Cell Signaling, CA, USA) at 4 °C which diluted 500 folds. Then, the membranes were washed in TBST three times followed by horseradish peroxidase (HRP)-labeled goat anti-rabbit/rat IgG (ZSGB-BIO, China). The specific protein bands were visualized with the ECL chemiluminescence detection kit (CWBIO), followed by exposure to Kodak X-ray films.

Detection of apoptosis and cell cycle distribution by flow cytometry

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 h, processed for labeling with propidium iodide, and kept in the dark for 30 min. The samples were loaded into a flow cytometer (Beckman Coulter, Kraemer Boulevard Brea, CA, USA). Cells were analyzed at a rate of 200–500 cells/s using FACS flow as at the sheath fluid, and 10,000 events were recorded for each sample, and the percentages of G0/G1-, S-, and G2/M-phase cells were calculated with Gallios™ software.

Statistical analysis

SPSS 17.0 statistical software was used for statistical analysis. Statistical comparisons of mean values were calculated using the two-sided Student's *t* test with the assumption of unequal variances. $P<0.05$ was considered significant. All data were presented as mean ± SD.

Results

Characterization of INPP4B expression in MDA-MB-231 cells

Our first aim was to evaluate the levels of INPP4B expression in MDA-MB-231 cells. MCF-7 cells which have been proved to have high levels of INPP4B were used as positive controls. Result showed that INPP4B in MDA-MB-231 cells presented a low-level expression or nonexpression, utilizing DNA agarose gel electrophoresis or western blotting method (Fig. 1a, b).

Overexpression of INPP4B in MDA-MB-231 cells via lentivirus transfection

The Lenti-INPP4B was constructed to infect MDA-MB-231 cells, and Lenti-GFP was used as a negative control to reflect transfection efficiency indirectly. Results showed that transfection efficiency reached 80 % in the group of MOI = 20, and this group was selected as the fixed single group for use in following treatments (Fig. 2a). To confirm the expression of INPP4B at the mRNA and protein levels, real-time PCR and western blotting were performed respectively. As shown in Fig. 2b, INPP4B mRNA expression in Lenti-INPP4B group was about 110 times higher than in the control/Lenti-GFP group at MOI of 20 ($P=0.00286$). Values were normalized against the internal control GAPDH and expressed as a fold overexpression above the value for MDA-MB-231 cells. Consistently, similar results were obtained by western blotting analysis of INPP4B protein. The noticeable strips around 105 kD in the Lenti-INPP4B group were in accordance with the size of INPP4B molecular weight, while no INPP4B-specific strips around 105 kD appeared in the other groups (Fig. 2c). This indicated that lentivirus system was effective to overexpress INPP4B in MDA-MB-231 cells.

Influence of INPP4B overexpression and PARP inhibitor AG014699 combination treatment on the level of p-AKT

It has been reported that the decreased INPP4B expression could enhance AKT phosphorylation and tumorigenic potential. Western blotting analysis showed that levels of p-AKT were significantly decreased upon treatment with overexpression of INPP4B. Furthermore, our results also demonstrated that PARP inhibitor AG014699 increased p-AKT levels in a dose-dependent manner, and when INPP4B was overexpressed in the AG014699-treated cells, the levels of p-AKT reduced significantly compared with cells treated by AG014699 alone (Fig. 3).

Antitumor effect of INPP4B overexpression on MDA-MB-231 cells

Growth curve of virus-infected MDA-MB-231 cells (from days 1 to 5) was detected by CCK-8. A remarkable drop in growth rate was observed when comparing the Lenti-INPP4B group with the control/Lenti-GFP group. This difference

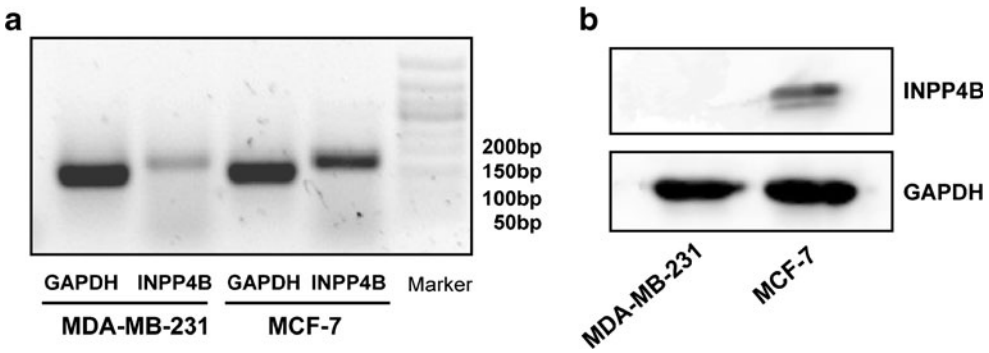

Fig. 1 INPP4B in MDA-MB-231 cells presented a low expression level. **a** DNA gel electrophoresis showed that the mRNA of INPP4B gene in MDA-MB-231 cells presented a low expression. MCF-7 cells which have been proved to have a high level of INPP4B expression were used as a positive control. **b** Western blotting was used to confirm that there is no expression in MDA-MB-231 cells

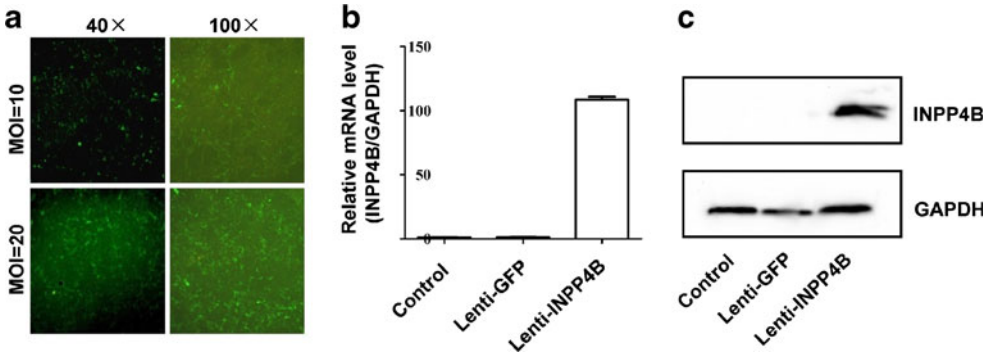

Fig. 2 Overexpression of INPP4B in MDA-MB-231 cells via lentivirus transfection. **a** To test the optimal virus titer, we set up two groups, with multiplicity of infection MOI of 10 and 20. Magnification is 40 and 100, respectively. The transfection efficiency reached 80 % in the group of MOI = 20, and this group was selected as the fixed single group for use in the following treatments. **b** Real-time PCR assay was performed to assess INPP4B mRNA expression, and the relative INPP4B level was counted by formula $2^{-\Delta\Delta Ct}$ in MDA-MB-231 cells. INPP4B mRNA expression in Lenti-INPP4B group was about 110 times higher than that in the control/Lenti-GFP group at MOI of 20. **c** Western blotting result showed that the lentivirus made MDA-MB-231 cells overexpress INPP4B successfully. The noticeable strips around 105 kD in the Lenti-INPP4B group were in accordance with the size of INPP4B molecular weight, while no INPP4B-specific strips around 105 kD appeared in other groups. All the data from at least three independently repeated experiments were expressed as mean ± SD

increased gradually as the time went on ($P<0.05$; Fig. 4). Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) flow cytometric analysis showed that the sum of early and late apoptosis rates was 2.85 and 5.48 %, respectively, in the control group and Lenti-INPP4B group, indicating that INPP4B overexpression did not increase apoptosis rate but rather induced growth arrest ($P>0.05$; Fig. 8). As a result, the percentage of G1-stage cells in Lenti-INPP4B group was 74.54 %, as comparing with 62.86 % in the control group, indicating that INPP4B overexpression could block cells in G1 phase ($P<0.05$; Fig. 7b).

Antitumor effects of PARP inhibitor AG014699 on MDA-MB-231 cells

CCK-8 results showed that the inhibition effect of PARP inhibitor AG014699 on MDA-MB-231 cells was dependent on concentration. Under 0.1, 1, 10, 20, and 40 μM concentrations for 24 h, the cell viability was 94.67, 79.35, 63.07, 38.11, and 29.45 %, respectively (Fig. 5a), IC_{50}=17.77 μM. Ten micromoles was selected as fixed drug concentration combined with INPP4B overexpression. The inhibition effect of PARP inhibitor AG014699 on proliferation of MDA-MB-231 cells was dependent on time. Cell inhibition rate was 17.38, 27.49, and 59.67 %, respectively (Fig. 5b), after 8, 36, and 54 h. Annexin V-FITC/PI flow cytometric analysis showed that the sum of early and late apoptosis rates was 9.98, 53.10, and 62.38 %, respectively, in 1, 20, and 40 μM groups. These cells showed a gradual increase in apoptosis rate along with drug concentration increased ($P<0.05$; Fig. 5c). PI staining cycle distribution showed that the percentage of G2/M-stage cells were 60.37, 69.82, and 82.93 %, respectively, in 1, 20, and 40 μM groups, indicating that PARP inhibitor AG014699 could block cell cycle progression in G2/M phase ($P<0.05$; Fig. 5d).

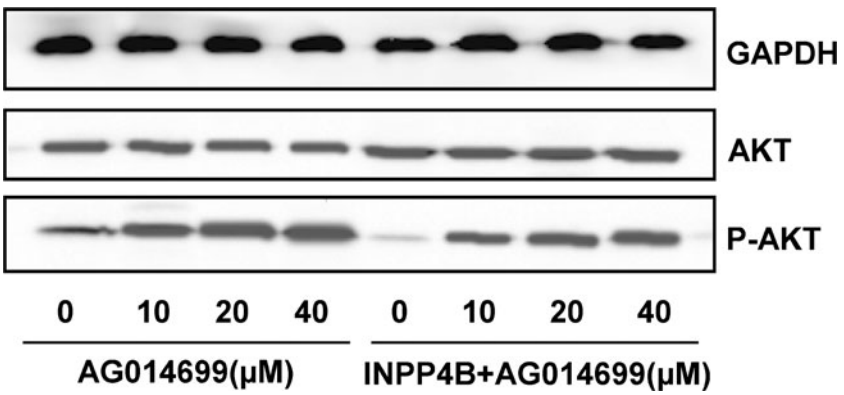

Fig. 3 Influence of INPP4B overexpression and PARP inhibitor AG014699 combination treatment on the level of p-AKT. *Left* MDA-MB-231 cells treated with different concentrations of the PARP inhibitors AG014699 10, 20, and 40 μM for 24 h and the expression of p-AKT were analyzed by western blotting. The results showed that levels of p-AKT were significantly increased in a dose-dependent manner. *Right* The levels of p-AKT after the combining treatment of INPP4B overexpression with PARP inhibitor AG014699 10, 20, and 40 μM. When INPP4B was overexpressed in the AG014699-treated cells, the levels of p-AKT reduced significantly compared with cells treated by AG014699 alone

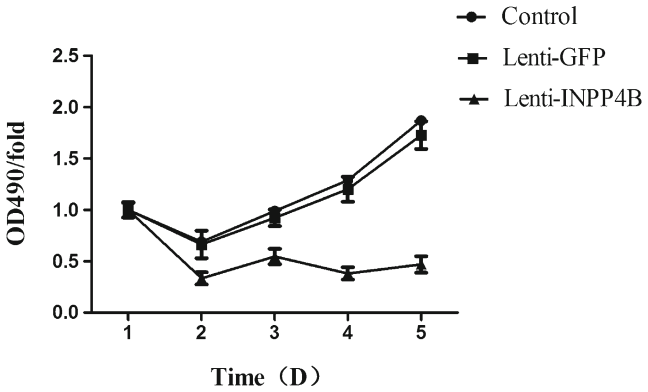

Fig. 4 Overexpression of INPP4B inhibited the proliferation of MDA-MB-231 cells. Growth curve of virus transfection cells (from days 1 to 5) was detected by CCK-8

Antitumor effects of the combination of INPP4B overexpression and PARP inhibitor AG014699 on MDA-MB-231 cells

The results above revealed that MDA-MB-231 cells were sensitive to either INPP4B overexpression or PARP inhibitor AG014699. Now, we wondered what would happen if the treatments were combined. Under the microscope, we found that cell quantity was significantly reduced, and the edge of cell membrane became folds in the group of INPP4B overexpression combined with PARP inhibitor AG014699 (Fig. 6). Next, the proliferation inhibition rate in combination treatment group was elevated to about 10 and 35 % compared with

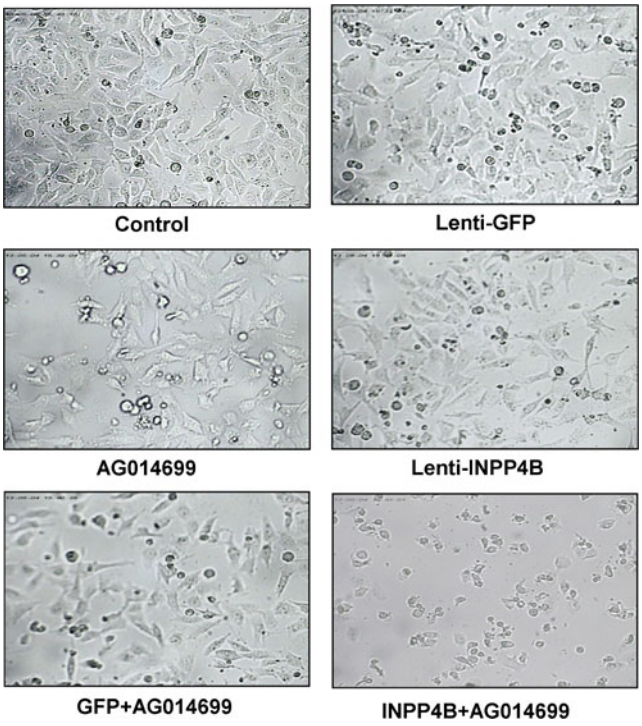

Fig. 6 MDA-MB-231 cells morphology alteration after treatment. MDA-MB-231 cells morphology was observed under optical microscope (×200). The number of MDA-MB-231 cells decreased significantly, and the cell membrane folds significantly after treatment of INPP4B gene overexpression combined with PARP inhibitor AG014699

INPP4B overexpression alone/PARP inhibitor AG014699 alone group after 24 h. The combination treatment showed

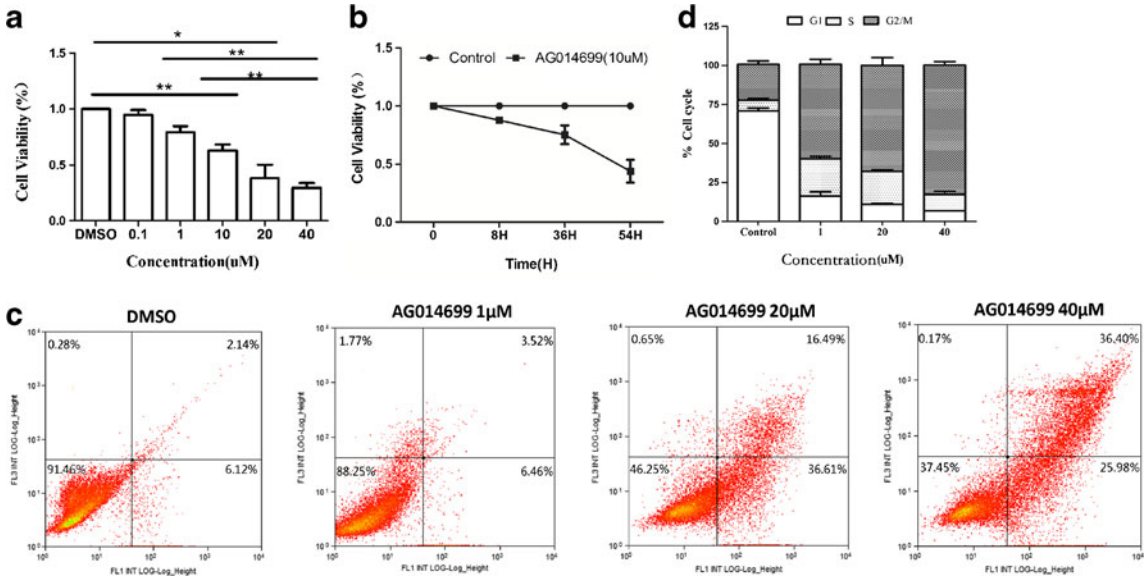

Fig. 5 Antitumor effects of PARP inhibitor AG014699 on MDA-MB-231 cells. **a** The doses for PARP inhibitor AG014699 were 0.1, 1, 10, 20, and 40 μM, respectively. The inhibition effect of PARP inhibitor AG014699 on MDA-MB-231 cells was dependent on concentration. **b** Viability MDA-MB-231 cells was measured at 8, 36, and 54 h after PARP inhibitor AG014699 (10 μM) treatment. The inhibition effect of PARP inhibitor AG014699 on proliferation of MDA-MB-231 cell was dependent on time. **c** Cells treated with PARP inhibitor AG014699 1, 20, and 40 μM showed a gradual drug concentration-dependent increase in apoptosis rate. **d** PARP inhibitor AG014699 could block cell cycle progression in G2/M phase. All the data from at least three independently repeated experiments were expressed as mean ± SD, which was analyzed by SPSS 17.0 statistical software (*p<0.05; **p<0.01)

potent antiproliferative effects in a time-dependent pattern. Cell inhibition rate was 29.21, 61.37, and 84.62 %, respectively, after 8, 24, and 36 h (Fig. 7a).

Annexin V-FITC/PI flow cytometric analysis showed that the sum of early and late apoptosis rates was 17.68 and 17.71 %, respectively, in the AG014699 group and combination treatment group. Our results showed that the combination treatment did not result in any further significant increase in apoptosis rate compared with PARP inhibitor AG014699 alone ($P>0.05$; Fig. 8).

Then, the cell cycle distribution was detected. PI staining cycle distribution showed that the G1 phase ratio was 72.54 and 72.62 %, respectively, in the Lenti-INPP4B group and combination treatment group. The combination treatment would not affect the cycle distribution which might transfer to G2/M, but cells would still be blocked in G1 phase (Fig. 7b).

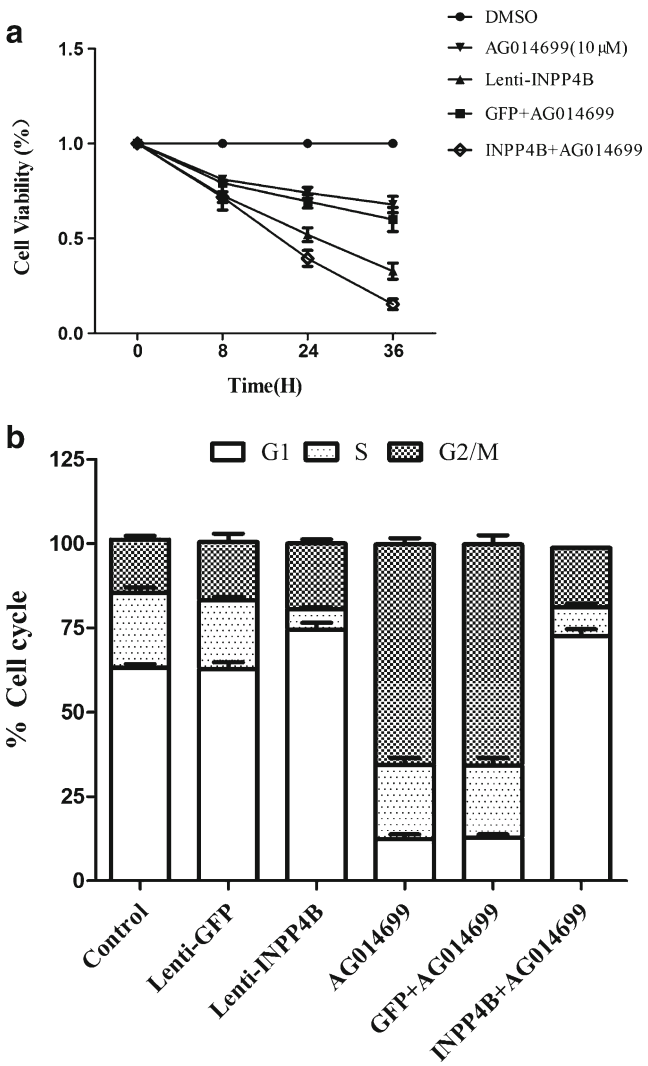

Fig. 7 Antitumor effects of combining treatment of INPP4B overexpression and PARP inhibitor AG014699. **a** The combination treatment exerted potent antiproliferative effects in a time-dependent pattern as shown in the CCK-8 assay. **b** Cell cycle distribution was assessed by flow cytometry. The combination treatment induced G1 phase cell cycle arrest compared with INPP4B overexpression and PARP inhibitor AG014699 (10 μM) alone (*$p<0.05$)

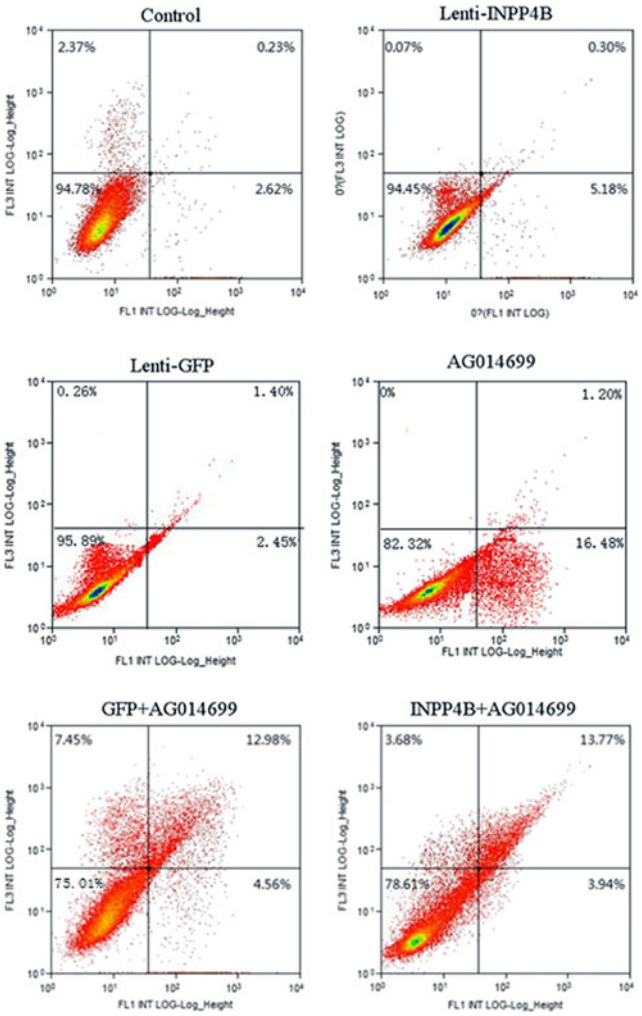

Fig. 8 Influence of different treatments on the apoptosis rate of MDA-MB-231 cells. Flow cytometric analysis was performed to evaluate the effect of the different treatments in apoptosis rate. We set up multiple groups: control, Lenti-GFP, AG014699, GFP + AG014699, Lenti-INPP4B, and INPP4B + AG014699. The combination of INPP4B overexpression and PARP inhibitor AG014699 treatment did not result in a further increase in apoptosis rate compared with PARP inhibitor AG014699 alone

Discussion

TNBC is a tumor subtype noted for more metastatic disease with an unfavorable prognosis [24, 25]. Decreased expression of INPP4B and overactivation of PI3K/AKT pathway is frequently observed in TNBC [26]. Activation of PI3K/AKT signaling pathway contributes to tumorigenesis, tumor metastasis, and angiogenesis [27-29]. Previous studies have shown that INPP4B protein expression was restricted to ER-positive

mammary ductal luminal epithelial cells and was lost in human basal-like/triple-negative breast cancers [5, 9]. Fedele et al. have assessed INPP4B protein expression in a panel of human breast cancer cell lines. INPP4B protein was expressed in ER-positive (MCF-7, T47D, and BT-474), but not ER-negative (MDA-MB-231, Hs578T, and BT-549) cell lines [5]. Our experiment further confirmed that TNBC cell line MDA-MB-231 presented a low-level INPP4B expression. Here, a lentivirus-based system was constructed which could effectively induce overexpression of INPP4B in MDA-MB-231 cells. INPP4B overexpression had a direct effect on suppressing cell proliferation with a time-dependent manner and inducing an accumulation G1 phase concomitant with a decreased S phase. We also found that INPP4B overexpression could decrease the levels of p-AKT. Arrest of proliferation might be associated with significantly suppressed activity of PI3K/AKT signaling pathway.

In circumstances where the HR-directed DNA repair pathway was deficient, inhibition of PARP could result in "synthetic lethality" [30]. Our experiment found that PARP inhibitor AG014699 showed significant antiproliferative effects in a dose- and time-dependent pattern on MDA-MB-231 cells. Moreover, as the AG014699 concentration increased, necrosis or apoptosis happened, and a G2/M cell cycle arrest was induced. However, clinical trials showed that the curative effect of PARP inhibitors was fluctuating even in BRCA1 or BRCA2 mutant tumors, and its effect has no relationship with platinum-based chemotherapy [31]. Recent research also suggests that PARP inhibitors induced activation of the PI3K/AKT pathway [22, 23]. In our study, PARP inhibitor AG014699 was shown to induce AKT phosphorylation in MDA-MB-231 cells, which caused activation of PI3K/AKT pathway and might partially attenuate the treatment effect and drive drug resistance happening.

BRCA1-dependent breast tumors, like all triple-negative breast tumors, harbor a variety of deregulated pathways, and it has been suggested that the management of these tumors with multiple targeted therapies may be a superior therapeutic approach [32-34]. The study by Kimbung et al. showed that co-targeting of the PI3K pathway improved the response of BRCA1-deficient breast cancer cells to PARP inhibitor [35]. Our research also supported this conclusion, and only the difference which was INPP4B overexpression was used to suppress PI3K pathway in combination with PARP inhibitor AG014699. Our results clearly indicated that the combination of INPP4B overexpression and PARP inhibitor AG014699 could further inhibit tumor growth and block cell cycle in G1 phase. It was interesting that the overexpression of INPP4B could antagonize the high levels of p-AKT caused by PARP inhibitor AG014699.

In recent years, it has been learned that combination treatments targeting different key pathways have the potential of both increasing the antitumor efficacy and reducing the risk of resistance. In the present study, our in vitro trials for the first time demonstrated that the combination of INPP4B overexpression and PARP inhibitor AG014699 with a more powerful antitumor effect was a potentially potent targeted therapy for breast cancers with PI3K signal pathway disorder and DNA repair deficiency, which would hopefully become an effective treatment method for the management of TNBC.

Acknowledgments This study was supported by a grant from the Shandong Province Science and Technology Development Project (2013GSF11839).

Conflicts of interest None.

References

1. Bauer KR, Brown M, Cress RD, Parise CA, Caggiano V. Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype: a population-based study from the California Cancer Registry. Cancer. 2007;109:1721–8.
2. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, et al. Triple-negative breast cancer: clinical features and patterns of recurrence. Clin Cancer Res. 2007;13:4429–34.
3. Haffty BG, Yang Q, Reiss M, Kearney T, Higgins SA, Weidhaas J, et al. Locoregional relapse and distant metastasis in conservatively managed triple negative early-stage breast cancer. J Clin Oncol. 2006;24:5652–7.
4. Agoulnik IU, Hodgson MC, Bowden WA, Ittmann MM. INPP4B: the new kid on the PI3K block. Oncotarget. 2011;2:321–8.
5. Fedele CG, Ooms LM, Ho M, Vieusseux J, O'Toole SA, Millar EK, et al. Inositol polyphosphate 4-phosphatase II regulates PI3K/Akt signaling and is lost in human basal-like breast cancers. Proc Natl Acad Sci U S A. 2010;107:22231–6.
6. Ferron M, Boudiffa M, Arsenault M, Rached M, Pata M, Giroux S, et al. Inositol polyphosphate 4-phosphatase B as a regulator of bone mass in mice and humans. Cell Metab. 2011;14:466–77.
7. Hodgson MC, Shao LJ, Frolov A, Li R, Peterson LE, Ayala G, et al. Decreased expression and androgen regulation of the tumor suppressor gene INPP4B in prostate cancer. Cancer Res. 2011;71:572–82.
8. Fu X, Osborne CK, Schiff R. Biology and therapeutic potential of PI3K signaling in ER+/HER2-negative breast cancer. Breast. 2013;22 Suppl 2:S12–8.
9. Gewinner C, Wang ZC, Richardson A, Teruya-Feldstein J, Etemadmoghadam D, Bowtell D, et al. Evidence that inositol polyphosphate 4-phosphatase type II is a tumor suppressor that inhibits PI3K signaling. Cancer Cell. 2009;16:115–25.
10. Won JR, Gao D, Chow C, Cheng J, Lau SY, Ellis MJ, et al. A survey of immunohistochemical biomarkers for basal-like breast cancer against a gene expression profile gold standard. Mod Pathol. 2013;26:1438–50.
11. McAuliffe PF, Meric-Bernstam F, Mills GB, Gonzalez-Angulo AM. Deciphering the role of PI3K/Akt/mTOR pathway in breast cancer biology and pathogenesis. Clin Breast Cancer. 2010;10 Suppl 3:S59–65.
12. Atchley DP, Albarracin CT, Lopez A, Valero V, Amos CI, Gonzalez-Angulo AM, et al. Clinical and pathologic characteristics of patients with BRCA-positive and BRCA-negative breast cancer. J Clin Oncol. 2008;26:4282–8.
13. Reddy KB. Triple-negative breast cancers: an updated review on treatment options. Curr Oncol. 2011;18:e173–9.

14. Turner NC, Reis-Filho JS. Basal-like breast cancer and the BRCA1 phenotype. Oncogene. 2006;25:5846–53.
15. D'Andrea AD, Grompe M. The Fanconi anaemia/BRCA pathway. Nat Rev Cancer. 2003;3:23–34.
16. Kinsella TJ. Understanding DNA damage response and DNA repair pathways: applications to more targeted cancer therapeutics. Semin Oncol. 2009;36:S42–51.
17. Yap TA, Sandhu SK, Carden CP, de Bono JS. Poly(ADP-ribose) polymerase (PARP) inhibitors: exploiting a synthetic lethal strategy in the clinic. CA Cancer J Clin. 2011;61:31–49.
18. Comen EA, Robson M. Poly(ADP-ribose) polymerase inhibitors in triple-negative breast cancer. Cancer J. 2010;16:48–52.
19. Kaelin Jr WG. The concept of synthetic lethality in the context of anticancer therapy. Nat Rev Cancer. 2005;5:689–98.
20. Farmer H, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature. 2005;434:917–21.
21. Park Y, Moriyama A, Kitahara T, Yoshida Y, Urita T, Kato R. Triple-negative breast cancer and poly(ADP-ribose) polymerase inhibitors. Anti-Cancer Agents Med Chem. 2012;12:672–7.
22. Chuang HC, Kapuriya N, Kulp SK, Chen CS, Shapiro CL. Differential anti-proliferative activities of poly(ADP-ribose) polymerase (PARP) inhibitors in triple-negative breast cancer cells. Breast Cancer Res Treat. 2012;134:649–59.
23. Szanto A, Hellebrand EE, Bognar Z, Tucsek Z, Szabo A, Gallyas Jr F, et al. PARP-1 inhibition-induced activation of PI-3-kinase-Akt pathway promotes resistance to taxol. Biochem Pharmacol. 2009;77:1348–57.
24. Kapp AV, Jeffrey SS, Langerod A, Borresen-Dale AL, Han W, Noh DY, et al. Discovery and validation of breast cancer subtypes. BMC Genomics. 2006;7:231.
25. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A. 2001;98:10869–74.
26. Bertucci MC, Mitchell CA. Phosphoinositide 3-kinase and INPP4B in human breast cancer. Ann N Y Acad Sci. 2013;1280:1–5.
27. Naumann RW. The role of the phosphatidylinositol 3-kinase (PI3K) pathway in the development and treatment of uterine cancer. Gynecol Oncol. 2011;123:411–20.
28. Wang YA, Johnson SK, Brown BL, McCarragher LM, Al-Sakkaf K, Royds JA, et al. Enhanced anti-cancer effect of a phosphatidylinositol-3 kinase inhibitor and doxorubicin on human breast epithelial cell lines with different p53 and oestrogen receptor status. Int J Cancer. 2008;123:1536–44.
29. Lopez-Knowles E, O'Toole SA, McNeil CM, Millar EK, Qiu MR, Crea P, et al. PI3K pathway activation in breast cancer is associated with the basal-like phenotype and cancer-specific mortality. Int J Cancer. 2010;126:1121–31.
30. Ashworth A. A synthetic lethal therapeutic approach: poly(ADP) ribose polymerase inhibitors for the treatment of cancers deficient in DNA double-strand break repair. J Clin Oncol. 2008;26:3785–90.
31. Tutt A, Robson M, Garber JE, Domchek SM, Audeh MW, Weitzel JN, et al. Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and advanced breast cancer: a proof-of-concept trial. Lancet. 2010;376:235–44.
32. Paez J, Sellers WR. PI3K/PTEN/AKT pathway. A critical mediator of oncogenic signaling. Cancer Treat Res. 2003;115:145–67.
33. Rakha EA, Reis-Filho JS, Ellis IO. Basal-like breast cancer: a critical review. J Clin Oncol. 2008;26:2568–81.
34. Cleator S, Heller W, Coombes RC. Triple-negative breast cancer: therapeutic options. Lancet Oncol. 2007;8:235–44.
35. Kimbung S, Biskup E, Johansson I, Aaltonen K, Ottosson-Wadlund A, Gruvberger-Saal S, et al. Co-targeting of the PI3K pathway improves the response of BRCA1 deficient breast cancer cells to PARP1 inhibition. Cancer Lett. 2012;319:232–41.