

Polarity Protein Par3 Sensitizes Breast Cancer to Paclitaxel by Promoting Cell Cycle Arrest.

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Abstract

Purpose Paclitaxel, belongs to tubulin-binding agents (TBAs), showed a great efficacy against breast cancer via stabilizing microtubules. Drug resistance limits its clinical application. Here we aimed to explore a role of Polarity protein Par3 in improving paclitaxel effectiveness.

Methods Breast cancer specimens from 45 patients were collected to study the relationship between Par3 expression and paclitaxel efficacy. The Kaplan–Meier method was used for survival analysis. Cell viability was measured in breast cancer cells (SK-BR-3 and T-47D) with Par3 over-expression or knockdown. The flow cytometry assays were performed to measure cell apoptosis and cell cycle. BrdU incorporation assay and Hoechst 33258 staining were performed to measure cell proliferation and cell apoptosis, respectively. Immunofluorescence was used to detect microtubule structures.

Results Par3 expression is associated with good response of paclitaxel in breast cancer patients. Consistently, Par3 overexpression significantly sensitizes breast cancer cells to paclitaxel by promoting cell apoptosis and reducing cell proliferation. In Par3 overexpressing cells upon paclitaxel treatment, we observed intensified cell cycle arrests at metaphase. Further exploration showed that Par3 overexpression stabilizes microtubules of breast cancer cells in response to paclitaxel, and resists to microtubules instability induced by nocodazole, a microtubule-depolymerizing agent.

Conclusion Par3 facilitates polymeric forms of tubulin and stabilizes microtubule structure, which aggravates paclitaxel-induced delay at the metaphase-anaphase transition, leading to proliferation inhibition and apoptosis of breast cancer cells. Par3 has a potential role in sensitizing breast cancer cells to paclitaxel, which may provide a more precise assessment of individual treatment and novel therapeutic targets.

Introduction

Breast cancer is the most commonly occurring malignant disease in women and the leading cause of cancer death among women.[1] Chemotherapy is the primary systemic treatment for breast cancer, by reducing the risk of recurrence and improving the prognosis of patients with breast cancer. Taxanes remain the cornerstone in the adjuvant and metastatic setting of breast cancer. Paclitaxel, one of the most commonly used taxanes, has shown great efficacy against breast cancer and is widely recognized as the first-line therapy.[2] Nevertheless, paclitaxel resistance is one of the major causes of treatment failure, and became a great obstacle in clinical applications in breast cancer.

Paclitaxel binds to β -tubulin in the α - β -tubulin heterodimer and stabilizes microtubules. It functions as a mitotic inhibitor by restraining spindle microtubule dynamics and causing a delay at the metaphase-anaphase transition during mitosis. Mechanisms related to the tubulin and the microtubule systems that mediate sensitivity to anti-mitotic are beginning to be unraveled.[3] Altered expression of microtubule-associated protein, such as overexpression of stathmin, decreased polymerization of microtubules, markedly decreased binding of paclitaxel, and then decreased sensitivity to paclitaxel in a panel of

human breast cancer cell lines.[4] β -tubulin mutations destabilized microtubule assembly and decreased paclitaxel binding to microtubules.[5] Specific β -tubulin isoforms differentially influence sensitivity to paclitaxel, for example, overexpression of class III β -tubulin is the most prominent mechanism of paclitaxel resistance in ovarian cancer.[6] In addition, extracellular signaling protein, such as TGFBI (transforming growth factor beta induced), may induce specific resistance to paclitaxel and mitotic spindle abnormalities in ovarian cancer cells, which was modulating by FAK- and Rho-dependent microtubules stabilization.[7]

Par3 (partitioning defective 3), a signaling scaffold protein in the Par3/Par6/aPKC (Par) complex contributing to the establishment and maintenance of cell polarity, appears to be a microtubule-associated protein, which directly regulates microtubule organization by promoting microtubule bundling and stabilization[8]. Spatially restricted cytoskeletal remodeling, regulated by polarity proteins, requires the cross-talk between polarity signaling and Rho GTPases.[9] During cell migration, Par complex was activated by integrin signaling and downstream CDC42 at inhibitory site of migration cells, leading to phosphorylation of GSK3 β , which ultimately blocks phosphorylation of APC. Unphosphorylated APC can bind to growing end of microtubules and stabilize the growing microtubules.[10–12] Par3 mediates cell protrusion through interaction with TIAM1 and regulates RAC GTPases activity, which are required where actin and microtubule reorganization takes place for the protrusive activity.[13, 14] Par3 also regulated microtubule dynamics at cell-cell contacts and proper positioning of the centrosome at the cell center via the interaction between its N-terminal and dynein.[15]

Emerging evidence points to an important role for Par proteins in regulating the loss of cell and tissue architecture in carcinoma.[16] Par3 has been reported both pro-oncogenic and tumor-suppressive activity depending on the tumor type.[17] During migration, Par3 regulates local microtubule dynamics and centrosome orientation, suggesting its potential function associated with the anticancer effect of paclitaxel.[15] Here we found that Par3 overexpressing cells treated with paclitaxel showed more stabilized microtubule structure and microtubule bundles, which promoting cell cycle arrest at metaphase, resulting in proliferation inhibition and cell apoptosis.

Materials And Methods

Patients and specimens

Total 45 breast cancer patients who were pathologically diagnosed with metastatic breast cancer and received paclitaxel-based chemotherapy as palliative treatment at Fudan University Shanghai Cancer Center (Shanghai, China) between Nov 2011 and Jan 2014, were enrolled in the study. This study was approved by the research ethical committee of Fudan University Shanghai Cancer Center, and informed consent was obtained from each patient before clinical data analyses. Patients received paclitaxel intravenously at a dose of 80 mg/m², on day 1, 8, and 15, every four weeks. Tumor assessment was performed at baseline, and then every two cycles until disease progression or death, according to the RECIST 1.1. Follow up was performed every three months for survival until death, contact failure, or the

end of the investigation, i.e., Jun 2015. Progression free survival (PFS) was defined as the time between the date of enrollment and the date of the earliest evidence of objective disease progression or death from any cause before documented disease progression. Clinical benefit rate (CBR) was defined as the percentage of patients with measurable disease at baseline who had the best objective tumor response of complete response (CR), partial response (PR) or stable disease (SD). Overall survival (OS) was defined as the time interval from enrollment to death. However, if death was not observed or patients were still alive at the last observation point, data would be censored.

Cell Lines And Reagents

SK-BR-3 and T-47D cells were purchased from Chinese Academy of Sciences Shanghai Branch Cell Bank (Shanghai, China). SK-BR-3 was maintained in Dulbecco's modified Eagle's medium (Life, USA) supplemented with 10% fetal bovine serum (Life, USA) at 37°C in a humidified incubator containing 5% CO₂. T-47D was maintained in RPMI-1640 medium (Life, USA) supplemented with 10% fetal bovine serum at 37°C in a humidified incubator containing 5% CO₂. Paclitaxel was purchased from Selleck (#S1150, USA).

Western Blot

Western blot

Total protein was extracted from breast cancer cells and tumor tissues in RIPA lysis buffer and separated in 8% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) before electro-transferred onto polyvinylidene difluoride (PVDF) membrane as previously described. The membrane was incubated with primary antibody overnight at 4°C. Subsequently, the membrane was incubated with secondary antibody for 1 h at room temperature. Immunoreactive bands on the membrane were visualized by the enhanced chemiluminescence assay. The primary antibodies to Par3 (11085-1-AP, 1:1000 dilution, Proteintech, China), α -tubulin (11224-1-AP, 1:5000 dilution, Proteintech, China) and β -actin (#3700, 1:3000 dilution, Cell signaling) were commercially obtained. The secondary monoclonal or polyclonal HRP-conjugated antibodies (1:10000) were purchased from Jackson ImmunoResearch (USA).

Immunofluorescence

Cells were seeded on glass coverslips in 24-well plates (1×10^5 per well). After 8 h, cells were treated with 0.7 μ M paclitaxel for 48h. In parallel, 1×10^5 cells per well were seeded in 24-well plates with glass coverslips and treated with PBS. Cells were fixed with 4% paraformaldehyde in PBS for 30 min, permeabilized in 0.2% Triton X-100 in PBS for 10 min, blocked with 1% BSA in PBS for 1h at room temperature (RT). Cells were then incubated overnight at 4°C with primary antibodies diluted in blocking reagent, washed three times with PBS for 10 min, and incubated in the appropriate fluorescently-conjugated secondary antibodies for 1 h at RT. Nuclei were counterstained with DAPI. Coverslips were

mounted in an anti-fade medium (DAKO) on glass slides, and examined by laser-scanning confocal microscopy. Primary antibodies included rabbit anti-Par3 (11085-1-AP, 1:500 dilution, Proteintech, China), mouse anti- β tubulin (66240-1-Ig, 1:500 dilution, Proteintech, China), rabbit anti- α tubulin (11224-1-AP, 1:500 dilution, Proteintech, China). Secondary antibody included goat anti-rabbit coupled with Alexa Fluor 594 (112-585-003, 1:500 dilution, Jackson ImmunoResearch, USA), goat anti-rabbit coupled with Alexa Fluor 647 (115-605-166, 1:500 dilution, Jackson ImmunoResearch, USA).

Measurement Of Soluble Tubulin Fraction

Cells were lysed in a microtubule stabilizing buffer containing 20mmol/l Tris-HCL (pH 6.8), 0.14mol/l NaCl, 0.5% NP40, 1mmol/l $MgCl_2$, 2 mmol/l EGTA and 4 μ g/ml paclitaxel as previously described. [7] The insoluble tubulin fraction was separated using centrifugation at 12,000 g for 10 minutes at 4 °C and the resulting pellet was resuspended using 1 \times SDS–PAGE loading buffer. Equal volumes of the soluble and insoluble fractions were loaded for western blotting as described. Membranes were probed using anti- α tubulin antibody which was detected using anti-mouse secondary antibodies.

Statistical analysis

Statistical analyses of clinical data were performed using SPSS 20.0 software. Pearson's Chi-square test was performed to analyze categorical variables. Student's t-test was used for comparison between groups. RFS and OS curves were depicted using Kaplan–Meier analyses (log-rank test). All the functional experiments were performed in triplicates, and results were presented as mean value \pm SD. The level of significance in the statistical analyses is indicated as * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Results

Par3 expression positively correlates with the paclitaxel efficacy in breast cancer patients.

Breast cancer specimens from 45 patients who had undergone curative resection and paclitaxel-based chemotherapy with matched adjacent normal breast tissues were collected to investigate the association between Par3 expression pattern and chemotherapeutic response of paclitaxel in breast cancer. Clinical characteristics of patients were listed in **Supplementary Table 1**. The typical immunostaining images were showed in Fig. 1A (**lower panel**). Chemotherapy-sensitive with clinical benefit (complete response [CR]/partial response [PR]/stable disease [SD] \geq 6) months was observed in 29 patients, while chemotherapy-resistant with no clinical benefit (SD \leq 6 months/progressive disease [PD]) was achieved in 13 patients (Fig. 1A and 1E). According to the median value of immunohistochemical staining scores of Par3, the patients were classified into Par3 low expression group and high expression group. The chi-square test was employed and the results indicated that low Par3 expression positively correlated with liver metastasis phenotypes ($P = 0.047$, **Supplementary Table 1**). High level of Par3 staining in tumor tissues was observed in 18 (18/29, 57.1%) patients reaching clinical benefit (CBR), but in 3 (3/13, 23.1 %) patients in non-CBR group (Fig. 1E). The Par3 expression in tumor tissues positively and significantly

correlated with the clinical response to chemotherapy ($P = 0.048$) (Fig. 1A and 1E). Par3 expression in tumor tissues of CBR groups was significantly higher than that in non-CBR groups (Fig. 1B). Moreover, low Par3 expression was significantly associated with shorter PFS ($P = 0.014$, Fig. 1C), compared with that observed in high expression group. The Par3 expression in the breast cancer was also negatively associated with OS, although without reaching statistical significance ($P = 0.48$, Fig. 1D).

Par3 decreases the viability and promotes the apoptosis of breast cancer cells in response to paclitaxel.

To study the role of Par3 in breast cancer cell viability, the endogenous Par3 expression was determined in eight breast cancer cell lines (Fig. S1A). For future experiments we employed SK-BR-3 and T-47D cell lines which representing human epidermal growth factor receptor 2 (HER2) + and luminal (hormone receptor [HR] + HER2-) breast cancer subtypes respectively, and their moderate expression made them viable to up- and down-regulate the expression of Par3 on one same cell line. We then over-expressed or knocked down Par3 in breast cancer cells SK-BR-3 and T-47D (Fig. S1B), and incubated the cells in medium with different concentration of paclitaxel. Cell viability was measured against a control group after 48 h and the results indicated that Par3 over-expression decreased cell viability whereas Par3 knockdown increased cell viability (Fig. 2A). The half-maximal inhibitory concentrations (IC₅₀) of paclitaxel on control and Par3 manipulated cells were then calculated and compared. The IC₅₀ values on Par3 over-expressed T-47D and SK-BR-3 cells were 3.5 nM (95%CI 1.1–14.9) and 185 nM (95%CI 76–390), respectively, which were dramatically decreased in comparison with the control groups (13.3 nM [95%CI 3.1–49.3] on T-47D and 460 nM [95%CI 154 – 127] on SK-BR-3) (Fig. 2A). Consistently, Par3 knockdown increased IC₅₀ of paclitaxel (56.9 nM [95%CI 17.8-194.3] on T-47D and 1400 nM [95%CI 427–4934] on SK-BR-3), approximately 2 or 6 folds higher than those of control cells (9.9 nM [95%CI 3.5–36.7] on T-47D and 795nM [95%CI 275–2452] on SK-BR-3) (Fig. 2A). These data propose that Par3 sensitizes breast cancer cells to paclitaxel.

The flow cytometry assays were then performed to measure the cell apoptosis in breast cancer cells with different Par3 expression. The results showed that paclitaxel induced apoptosis was significantly aggravated by Par3 over-expression but attenuated by Par3 knockdown in both SK-BR-3 and T-47D breast cancer cells (Fig. 2B and 2C). Similarly, the Hoechst staining detected 80% apoptotic cells in Par3 over-expressed SK-BR-3 cells, while Par3 knockdown significantly decreased the number of apoptotic cells (30%) (Fig. 2D and 2E). Furthermore, the regulators of apoptosis were determined by western blot and the results showed that Par3 over-expression strengthened paclitaxel induced alteration of apoptotic regulators, that is, the decrease of anti-apoptotic Bcl-2 protein, the increase of pro-apoptotic Bax protein, and the cleavage of PARP, caspase-9 and -3, were enhanced by Par3 over-expression (Fig. 2F and S2). In contrast, paclitaxel induced changes of apoptotic regulators were rescued by Par3 down-regulation (Fig. 2F and S2). Above results indicate that Par3 promotes cell apoptosis, thus increases the sensitivity of breast cancer cells to paclitaxel.

Par3 aggravates paclitaxel induced mitotic arrest at metaphase of breast cancer cells

To explore whether Par3 expression affect the proliferation of breast cancer cells, we first employed BrdU incorporation assay and found that Par3 over-expression decreased cell proliferation while Par3 knockdown promoted cell proliferation in SK-BR-3 cells subjected to paclitaxel (Fig. 3A). Next, flow cytometry results indicated that paclitaxel induced mitotic arrest in SK-BR-3 cells, which could be aggravated upon Par3 over-expression or reversed upon Par3 knockdown (Fig. 3B). We then determined the regulators of the cell cycle, cyclin B1 and A2. Cyclin B1 is the major mitotic cyclin partner. We detected an aggressive increase of cyclin B1 in paclitaxel treated group and Par3 up-regulation has a promoting role in cyclin B1 level (Fig. 3C). The increased levels of cyclin A2 in mammalian cells have been reported to delay metaphase and anaphase onset.[18, 19] Paclitaxel significantly decreased cyclin A2 level and Par3 exacerbated this effect (Fig. 3C and S3). Par3 knockdown showed a resistant role in the effect of paclitaxel (Fig. 3C and S3). These results indicate that upon paclitaxel, Par3 over-expression blocks more cells in M phase and then increases the sensitivity of tumor cells to paclitaxel (Fig. 3D).

To detail the promoting role of Par3 in paclitaxel induced mitotic arrest, we further stained α -tubulin of SK-BR-3 cells and observed microtubule structures (shown in red) undergoing marked morphological changes to mediate specific functions throughout the cell cycle (Fig. 3E and 3G). Over-expression of Par3 significantly increased metaphase cells, while Par3 knockdown decreased the cell number at metaphase (Fig. 3E and 3F). In paclitaxel treatment group, 60.20% cells at metaphase and a plus of Par3 over-expression elevated the cell number at metaphase to 70.89% (Fig. 3E and 3F). Consistently, Par3 knockdown lessened the cells at metaphase, which partially reversed the effect of paclitaxel (Fig. 3E and 3F). Moreover, a decrease of aneuploid cells was observed in Par3 over-expressing group while Par3 knockdown raised the numbers of aneuploid cells upon paclitaxel condition (Fig. 3G and 3H). Above data illuminate the promoting role of Par3 in paclitaxel induced mitotic arrest of breast cancer cells at metaphase.

Par3 stabilizes microtubules of breast cancer cells in response to paclitaxel

Paclitaxel is globally a microtubule-stabilizing drug which can interact with the microtubule system and function as antimetabolic agents. Par3 enhanced mitotic arrest might have an important role in paclitaxel sensitivity of breast cancer cells. To further investigate the mechanism that Par3 sensitized breast cancer cells to paclitaxel, we examined the functional link between Par3 expression and the stability of microtubules. When we treated breast cells SK-BR-3 with paclitaxel, β -tubulin immunostaining indicated the appearance of microtubule bundles (Fig. 4A and 4C). Remarkably, more, thick, rounded tubular structures were formed and higher fluorescence density were detected in Par3 over-expressed cells (Fig. 4A and 4C), indicating excessive microtubule assembly and stabilization. Consistently, microtubule bundles and β -tubulin staining density showed a significant decrease in Par3 knockdown cells (Fig. 4A and 4C).

We then employed nocodazole, a microtubule-depolymerizing agent, to confirm the role of Par3 in the regulation of microtubule stability. Upon nocodazole treatment, a substantial fraction of cells over-expressing Par3 possessed more stabilized microtubules (Fig. 4B and 4D). In contrast, Par3 knockdown

attenuated stabilized microtubules in nocodazole treated cells (Fig. 4B and 4D). Moreover, polymeric and soluble forms of α -tubulin were determined with western blot and the results indicated that Par3 over-expression significantly reduced the soluble α -tubulin, while Par3 knockdown raised the level of soluble α -tubulin (Fig. 4E and 4F). These results indicate that Par3 increases polymeric forms of α -tubulin and stabilizes microtubule structure, which might be involved in the chemotherapeutic response of paclitaxel in breast cancer.

Discussion

We presently found breast cancer patients with higher Par3 expression respond well to paclitaxel-based chemotherapy and Par3 sensitized breast cancer cells to paclitaxel-induced decrease of cell viability, indicating the potential role of Par3 in enhancing paclitaxel chemosensitivity in breast cancer.

Par3, along with Par6 and Ser/Thr kinase atypical PKC (aPKC), forms Par apical module and is an evolutionarily conserved component of a common genetic pathway involved in cell polarity establishment and maintenance. It contains three PSD-95/Discs-large/ZO-1 (PDZ) domains, an amino-terminal dimerization domain and a carboxy-terminal aPKC interaction domain.[20, 21] These domains bind multiple proteins including junctional adhesion molecules and nectin,[22, 23] phospholipids (PIP2), PTEN (the phosphatase and tensin homologue),[24–26] and the Rac-GEF Tiam1,[27, 28] thereby coupling polarity establishment to Rac activation. Par3 depletion activated atypical PKC-dependant JAK/Stat3, induced MMP9, destroyed the extracellular matrix, and promoted the invasion of breast cancer cells.[29] Moreover, loss of Par3 promoted metastatic behavior of ErbB2-induced breast cancer cells by inhibiting E-cadherin junction stability, disrupting membrane and actin dynamics at cell-cell junctions and decreased cell-cell cohesion in a manner dependent on the Tiam1/Rac-GTP pathway.[30] Recently, we found that Par3 interacted with ZO-1, a component in tight junctions (TJ), and promoted the metastasis bladder cancer via GSK-3 β /Snail/Par3/ZO-1 axis.[31] These results suggest various roles of Par3 in tumor development and further investigations of Par3 regulating the chemosensitivity of breast cancer are warranted.

Paclitaxel belongs to tubulin-binding agents (TBAs) and disrupts the mitotic spindle by stabilizing microtubule structure, which does not satisfy the spindle assembly checkpoint, thus leads to a tight metaphase arrest, and eventually induces either cell death through apoptosis or mitotic slippage (exit from mitosis into interphase without cell division).[3, 32, 33] Microtubule dynamics plays an important role in taxane binding and exerting its anti-tumor activity, and many reported factors that decrease the microtubule stability induce taxane resistance. Some microtubule-associated proteins (MAPs), such as tau, MAP2 and MAP4, directly bind to and stabilize microtubules against depolymerization while other MAPs, such as stathmin, decreased polymerization of microtubules. Ahmed et al. reported that TGFBI showed integrin-dependent regulation of paclitaxel sensitivity via focal adhesion kinase (FAK) - and Rho-dependent stabilization of microtubules.[7]

Par3 also appeared to be a microtubule-associated protein, which bound, bundled and stabilized microtubules through direct binding to microtubules via its N-terminal portion.[8] Consistently, our data showed that Par3 increased polymeric forms of tubulin and stabilized microtubule bundles, and its over-expression dramatically aggravated paclitaxel induced assembling of microtubule bundles and sensitizing the breast cancer cells to paclitaxel-induced apoptosis, eventually improved the therapeutic effect of paclitaxel. Moreover, Par3 stabilized the microtubule not only through its direct binding, but also through signaling processes and its effects on other cytoskeletons, such as actin. Rho GTPases is crucial for cytoskeletal regulation and changes or regulators of the actin cytoskeleton can mediate sensitivity to TBAs.[3] Par3 interacted with TIAM1 and regulates RAC GTPases activity, and reorganized actin and microtubule during migration. Par3 has been reported regulating actin cytoskeleton by controlling the morphogenesis of dendritic spines, cellular structures largely supported by filamentous actin.[34] Par3 could also associate with dynein and contributed to the local regulation of microtubule dynamics at cell-cell contacts and proper positioning of the centrosome at the cell center.[15] Compared with other factors, Par3 exerted an integrated, multilevel regulation of microtubule dynamics and may be a more critical regulatory factor for paclitaxel resistance.

The effectiveness of paclitaxel is limited by various side effects associated with its use.[35, 36] A reduce of the paclitaxel dose by 20% may alleviate some of the neuropathic side effects, one of the major side effects of paclitaxel.[37] We found that Par3 over-expression dramatically decreased IC50 of paclitaxel in breast cancer cells, proposing a potential role of Par3 in the improvement of the clinical application of paclitaxel. Our previous study showed that transcription factor Sp1 regulates Par3 expression via binding with *PARD3* promoter in breast cancer.[38] Promoting Par3 expression by inducing Sp1 may potentially increase the sensitivity of paclitaxel in breast cancer. Though the samples were too few to draw a definitive conclusion, we would predict the beneficial role of Par3 in breast cancer patients treated with paclitaxel.

Conclusion

We describe here a mechanism to improve paclitaxel sensitization via Par3 mediated microtubules stability. Par3 increases polymeric forms of tubulin and stabilizes microtubule structure, which aggravate paclitaxel-induced delay at the metaphase-anaphase transition, resulting in proliferation inhibition and apoptosis of breast cancer cells. It suggests a positive role of Par3 to improve the clinical application of paclitaxel.

Declarations

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Conflict of interest All authors declared that they had no conflicts of interest..

Availability of data and material The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Author contributions She Chen, Yingying Xu and Biyun Wang designed the study. Yannan Zhao, Huitong Peng, Limiao Liang, Yi Li, Xichun Hu collected the clinical data, performed the experiments, created the figures and tables. Yannan Zhao, Yingying Xu and She Chen wrote and edited the manuscript.

Ethics approval This study was approved by the research ethical committee of Fudan University Shanghai Cancer Center.

Consent to participate and publication The consent was obtained from each patient before clinical data analyses.

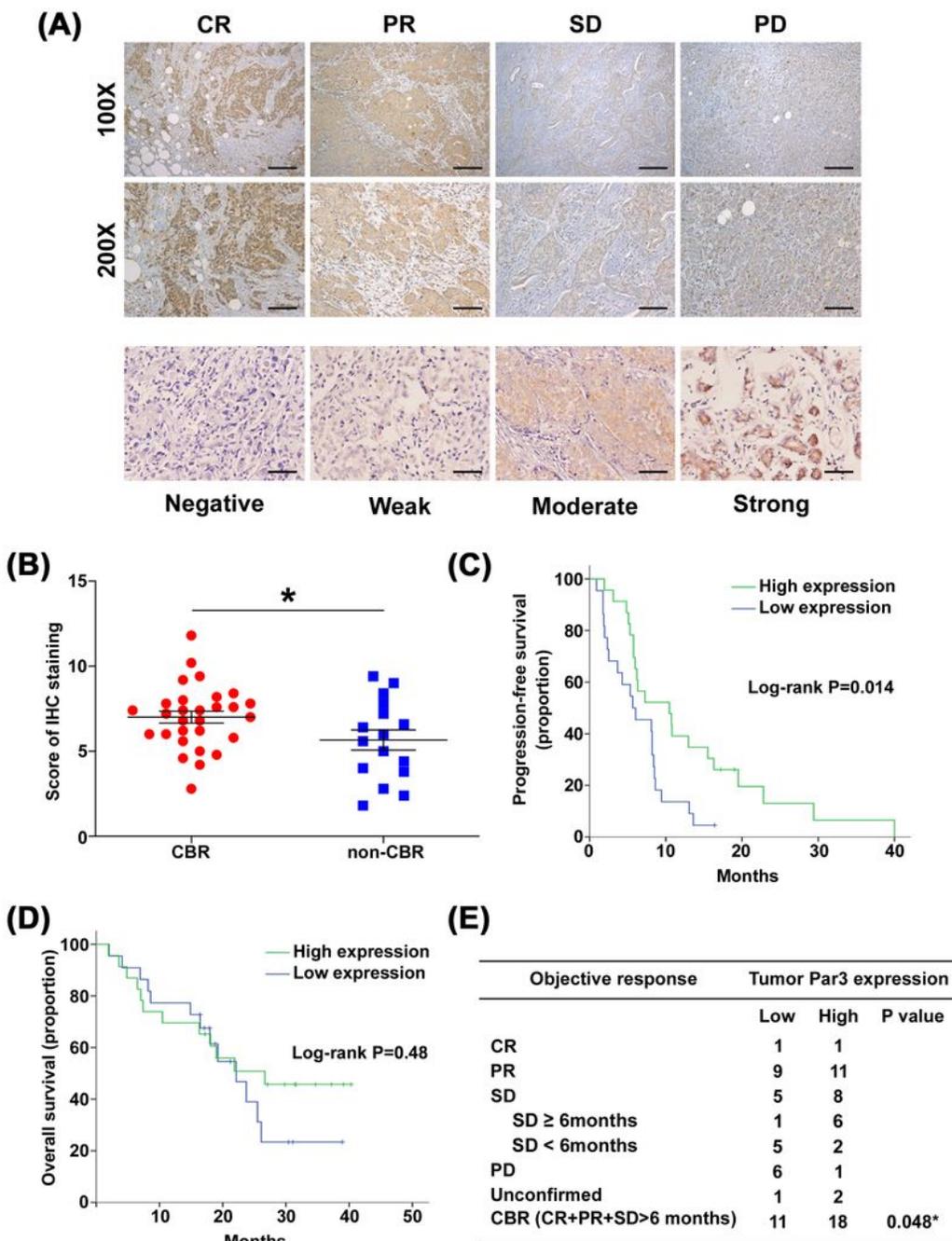
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Figures

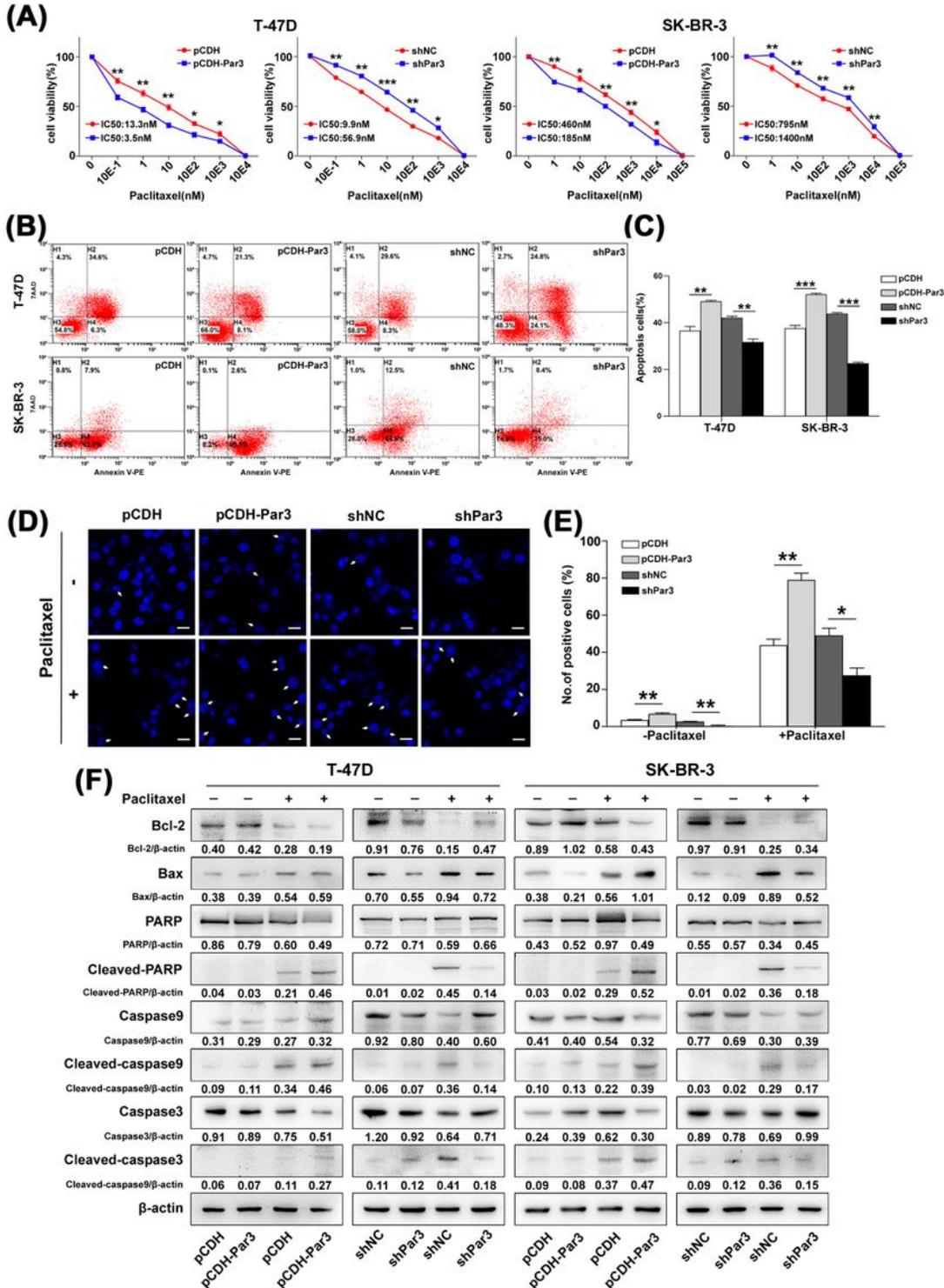


Zhao et al. Figure 1

Figure 1

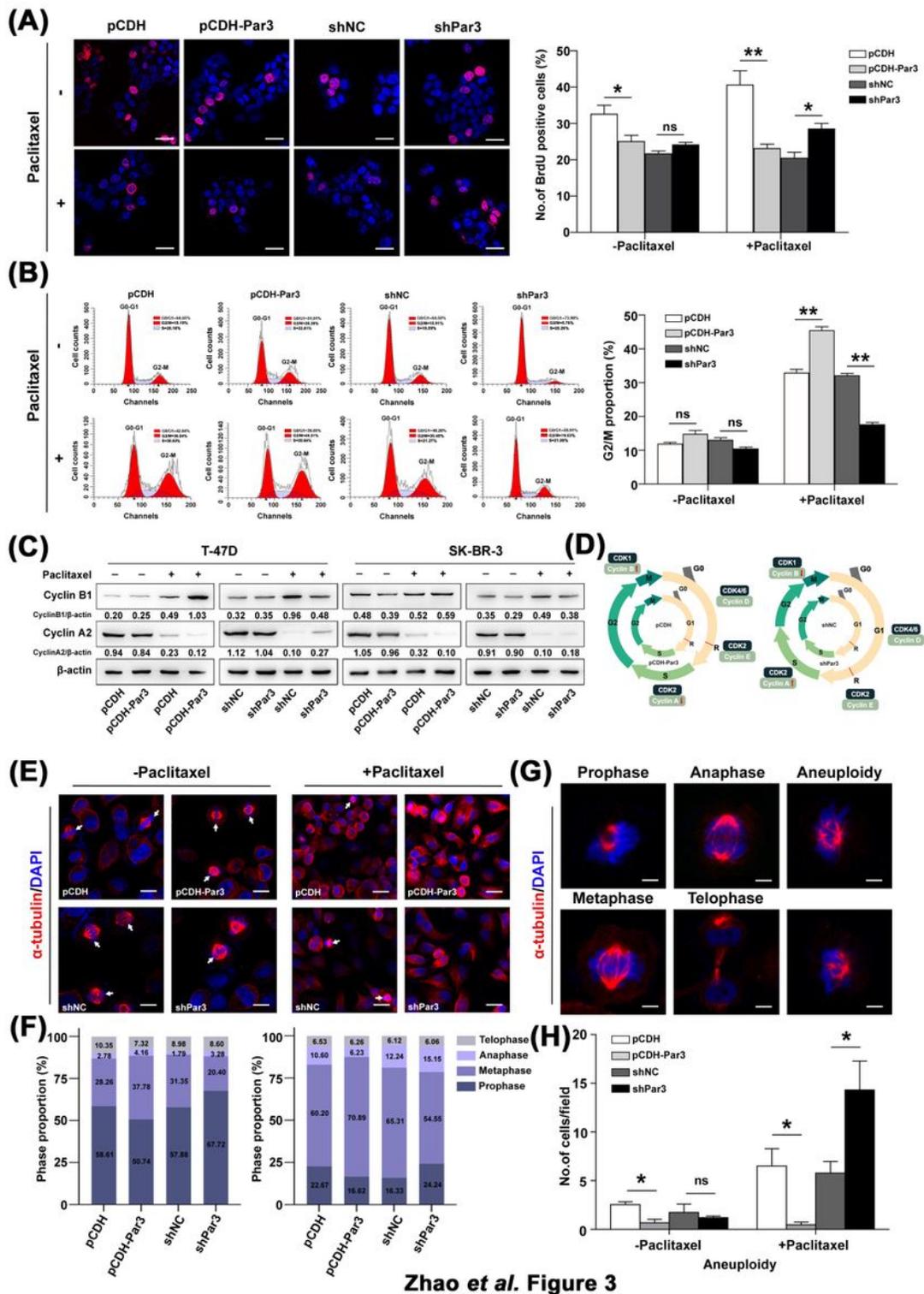
Par3 expression positively correlates with the paclitaxel efficacy in breast cancer patients. (A) Typical patterns of Par3 staining in tumor tissues of 45 breast cancer patients with paclitaxel response. The breast cancers with complete response (CR)/partial response (PR)/stable disease (SD) \geq 6 months responses to paclitaxel-based chemotherapy expressed higher levels, whereas those with stable disease (SD) $<$ 6 months/progressive disease (PD) responses expressed lower levels of Par3 (upper panel). The

typical immunostaining images of Par3 (lower panel). Scale bars: 100 \times , 100 μ m; 200 \times , 50 μ m. (B) Scores of immunochemistry staining of Par3 in 45 breast cancer patients with different chemotherapeutic response. *P < 0.05. (C) Kaplan-Meier analysis of progression-free survival (PSF) for Par3 expression in breast patients undergone paclitaxel-based chemotherapy. (D) Kaplan-Meier analysis of overall survival (OS) for Par3 expression in breast patients undergone paclitaxel-based chemotherapy. (E) Correlation of chemotherapeutic response and clinicopathologic features with Par3 expression.



Zhao et al. Figure 2

Par3 decreases the viability and promotes the apoptosis of breast cancer cells in response to paclitaxel. (A) Par3 over-expression decreases cell viability while Par3 knockdown increases cell viability of T47D and SK-BR-3 cells with paclitaxel treatment. Par3 were over-expressed or knocked down in SK-BR-3 and T-47D cells. Cells were then incubated in medium with different concentration of paclitaxel. Cell proliferation was measured using the Cell Counting Kit-8. The half-maximal inhibitory concentrations (IC50) of paclitaxel were calculated and compared against a control group. *P < 0.05, **P < 0.01, ***P < 0.001. (B-E) Par3 over-expression increases cell apoptosis while Par3 knockdown decreases cell apoptosis of T47D and SK-BR-3 cells upon paclitaxel treatment. Cells were treated with paclitaxel for 48 h. Early apoptosis was estimated using apoptosis detection kit and 7-AAD (B). Apoptosis cells were calculated and compared against a control group (C). The apoptotic cells were detected with Hoechst staining (D), estimated and compared against a control group (E). Scale bars represent 20 μ m. *P < 0.05, **P < 0.01, ***P < 0.001. (F) Par3 over-expression strengthens paclitaxel-induced alteration of apoptotic regulators while Par3 down-regulation rescues paclitaxel-induced changes of apoptotic regulators. T47D and SK-BR-3 cells were treated with paclitaxel for 48 h. Cell lysates were subjected to western blot. The apoptotic regulators were detected and normalized against β -actin.



Zhao *et al.* Figure 3

Figure 3

Par3 aggravates paclitaxel induced mitotic arrest at metaphase of breast cancer cells. (A) Par3 expression promotes the proliferation of breast cancer cells. SK-BR-3 cells were treated with paclitaxel for 48 h and cell proliferation was estimated with BrdU incorporation assay (left panel). Scale bars represent 20 μ m. BrdU positive cells were detected, calculated and compared against a control group (right panel). * $P < 0.05$, ** $P < 0.01$. (B) Paclitaxel induces cell cycle arrest at G2/M phase could be aggravated upon

Par3 over-expression or reversed upon Par3 knockdown. SK-BR-3 cells were treated with paclitaxel for 48 h. Flow cytometric analysis were performed to measure the cells at different phases of cell cycle (left panel). Cell proportion at G2/M phase was compared against a control group (right panel). **P < 0.01. (C) Par3 alteration affects protein expression of the major regulator of cell cycle. T47D and SK-BR-3 cells were treated with paclitaxel for 48 h. Cell lysates were subjected to western blot. Cyclin B1 and A2 were detected and normalized against β -actin. (D) A model of cell cycle regulated by Par3 alteration upon paclitaxel. Par3 up-regulation promotes the protein level of cyclin B1, a marker of mitotic phase, and decreases the protein level of cyclin A2, a regulator delaying metaphase and anaphase. In contrast, Par3 down-regulation decreases cyclin B1 protein but increase cyclin A2 protein. (E-F) Par3 promotes paclitaxel induced mitotic arrest. SK-BR-3 cells were cultured with paclitaxel for 48 h and stained with antibody specific to α -tubulin. The microtubule structures (shown in red) undergoing metaphase were marked with white arrows (E). Scale bars represent 20 μ m. Cell proportion at marked morphological changes were calculated and shown (F). (G) Typical patterns of α -tubulin staining in cells undergoing marked morphological changes. Scale bars represent 10 μ m. (H) Par3-expression decreases aneuploid cells. SK-BR-3 cells were stained with antigen specific to α -tubulin. The aneuploid cells counted and compared to a control group. *P < 0.05, **P < 0.01.

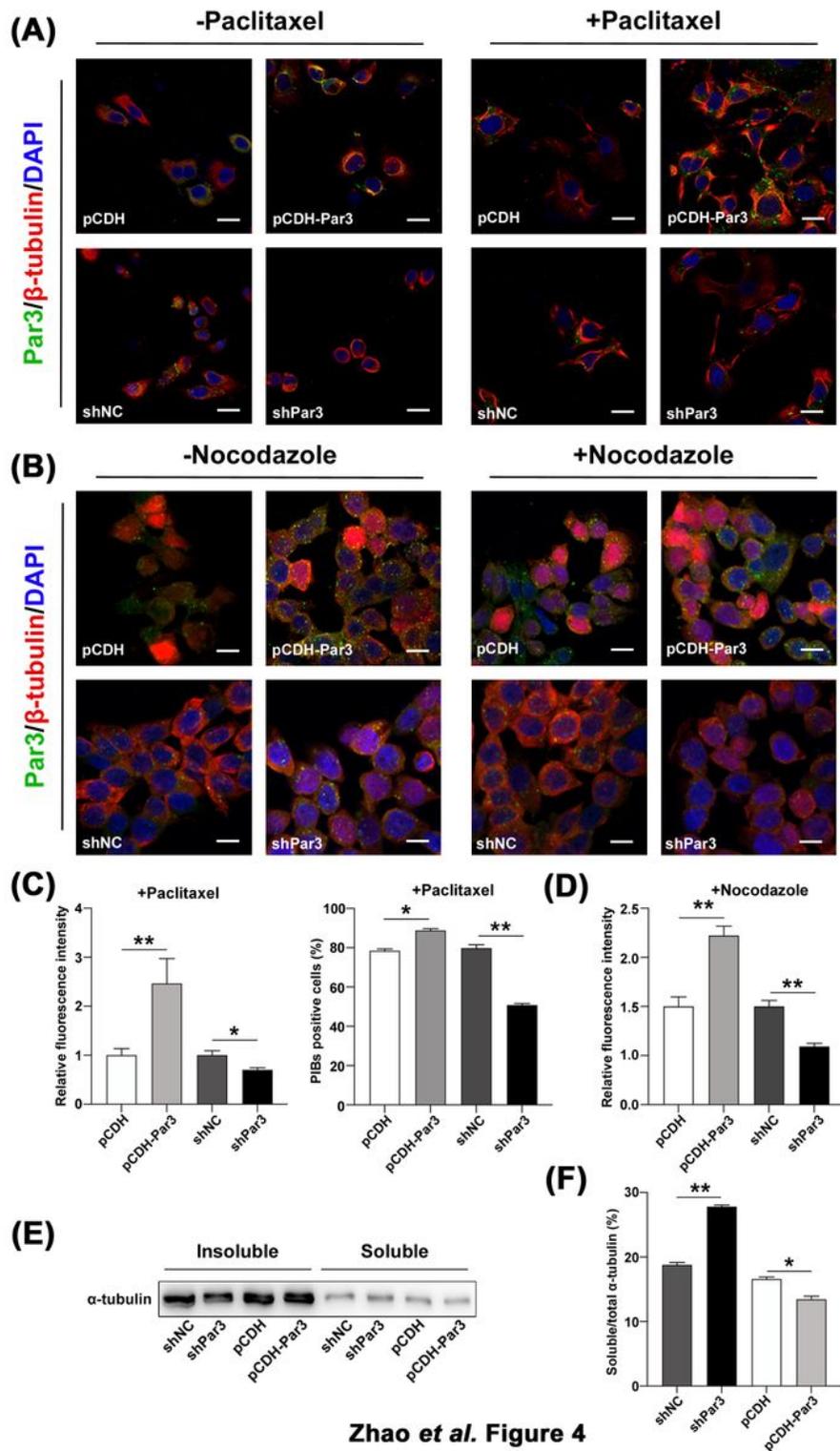


Figure 4

Par3 stabilizes microtubules of breast cancer cells in response to paclitaxel. (A and C) Par3 promotes microtubule bundling of breast cancer cells with paclitaxel treatment. SK-BR-3 cells with Par3 up-regulation or down-regulation were cultured with paclitaxel for 48 h and stained with antigen specific to β -tubulin (red). Scale bars represent 20 μ m (A). Relative fluorescence intensity of β -tubulin immunostaining (left panel) and PIBs (paclitaxel induced bundles) positive cells (right panel) were shown (C). * $P < 0.05$,

**P < 0.01. (B and D) Par3 stabilizes microtubules of breast cancer cells with nocodazole treatment. SK-BR-3 cells with Par3 up-regulation or down-regulation were cultured with nocodazole for 48 h and stained with antigen specific to β -tubulin (red). Scale bars represent 20 μ m. (B). Relative fluorescence intensity of β -tubulin immunostaining was shown (D). *P < 0.05, **P < 0.01. (E-F) Par3 reduces the soluble forms of α -tubulin. Cells were lysed and soluble and insoluble tubulin fraction was separated. Equal volumes of soluble and insoluble fractions were then loaded for western blotting with α -tubulin antibody (E). The percent of soluble α -tubulin were shown and compared to a control (F). *P < 0.05, **P < 0.01.

Supplementary Files

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