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Original Research

PFKFB3 potentially contributes to paclitaxel resistance in breast cancer cells through TLR4 activation by stimulating lactate production

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Abstract: Paclitaxel is a commonly used agent for breast cancer therapy, which comes across the obstacle "drug resistance", resulting in shortened overall survival of patients. Warburg effect has become one character of cancer cell and was reported to induce paclitaxel resistance, the mechanism of which is poorly understood. In this study, we sought to examine the role of 6-Phosphofructo-2-kinase (PFKFB3), a critical regulator of glycolysis, in paclitaxel resistance development. Two clones of paclitaxel resistant breast cancer cells, MCF-7^{R}A and MCF-7^{R}B, were established by a long term exposure of MCF-7 cells to paclitaxel. Consequently, PFKFB3 expression was found to be increased in MCF-7^{R}A and MCF-7^{R}B cells compared with MCF-7 cells. Silencing PFKFB3 expression markedly reduced the IC50 concentrations of MCF-7^{R}A and MCF-7^{R}B cells. Moreover, PFKFB3 modulated toll like receptor 4 (TLR4) and MyD88 expression as well as interleukin (IL)-6 and IL-8 release from breast cancer cells in response to paclitaxel exposure. In addition, PFKFB3 overexpression boosted up fructose-2,6-bisphosphate (F2,6BP) and lactate production. The enhanced lactate contributed to TLR4 signaling activation, IL-6 and IL-8 generation, and cell viability promotion in MCF-7 cells. In all, we characterized the novel role of PFKFB3 in induction of paclitaxel resistance by raising lactate production and activating TLR4 signaling.

Key words: Breast cancer, paclitaxel resistance, Warburg effect, 6-Phosphofructo-2-kinase, toll like receptor 4, lactate.

Introduction

Breast cancer is one of the most deadly diseases diagnosed in females (1). In 2008, it has been reported that 1.39 million women suffered from breast cancer, which caused 458,400 women to death around the world (1). China has a low incidence of breast cancer, but its occurrence, especially in urban areas, has augmented up to twice as fast as global rates since the 1990s (2). In China, there were 169 452 new cases diagnosed with breast cancer and 44 908 related deaths by 2008, accounting for 12.2% of global cases and 9.6% of related deaths (3).

In clinical treatment of breast cancer, paclitaxel and its derivatives are taken as the most effective and active medicine (4). Paclitaxel can suppress cancer cells mitosis by inhibiting microtubule dynamics during cell division (5). Despite its wide use in cancer therapy, amounts of reports have concerned on drug resistance in response to paclitaxel. Molecular mechanism relating to the formation of paclitaxel resistance has been characterized by some reports. P-glycoprotein in has been associated with multi-drug resistance, including paclitaxel resistance (6). Furthermore, Glutathione-S-transferase, glutathione peroxidase, thymidylate synthase, and multidrug resistance protein-1 have also been demonstrated to be implicated in paclitaxel resistance (7). Hypoxia inducible factors, highly expressed in breast cancer, contribute to the resistance of breast cancer stem cells to paclitaxel in vitro and in vivo (8). Additionally, the expression of tissue inhibitor of metalloproteinase (TIMP-1) as well as the state of epithelial–mesenchymal transition (EMT) is considered as critical causes in paclitaxel resistance (9, 10).

Cancer cells majorly rely on aerobic glycolysis instead of mitochondrial oxidative phosphorylation for energy production, a phenomenon called "Warburg effect" (11). Recently, Warburg effect was shown to play an essential role in the development of paclitaxel resistance (12). The glycolytic pathway is regulated by multiple substrates and enzymes, which include Phosphofructokinase-1 (PFK-1), the rate-limiting enzyme of glycolysis (13, 14). Fructose-2,6-bisphosphate (F2,6BP) is a potent allosteric activator of PFK-1, resulting in high activity of PFK-1 (15). Cellular levels of F2,6BP are controlled by the activity of the bifunctional enzyme 6-Phosphofructo-2-kinase (PFKFB3) (16). Evidence has emerged that PFKFB3 is involved in cell immortal and malignant transformation. Indeed, PFKFB3 is required for ras induced transformation in mice fibroblast (17). Moreover, PFKFB3 expression was found to be upregulated in multiple malignant neoplasms, including colon, breast, ovarian, and thyroid carcinomas (18). Several mitogenic stimuli, such as progesterone, serum, and insulin, may also induce PFKFB3 expression (19-21). In breast cancer cells, the estrogen receptor directly promotes PFKFB3 mRNA transcription which influences the glycolysis and cell viability (22). However, the role of PFKFB3 in paclitaxel resistance has not been elucidated.

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In the current study, we sought to delineate whether PFKFB3 is involved in the induction of paclitaxel resistance in breast cancer. We established two clones of breast cancer cells in resistant to paclitaxel and found that PFKFB3 was overexpressed in these cells. Silencing PFKFB3 expression enhanced drug sensitivity of these cancer cells and decreased their IC50 concentrations. Furthermore, our data showed that PFKFB3 promoted toll like receptor 4 (TLR4) signaling activation in response to paclitaxel conditioning via raised lactate production.

Materials and Methods

Cell culture and treatment

The breast cancer cell line, MCF-7 was purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Invitrogen) under a 37°C, 5% CO_2 circumstance.

To established paclitaxel resistant cell line, MCF-7 cells were exposed to increasing paclitaxel concentration in the culture medium over 4 months. Paclitaxel concentration was enhanced in a stepwise manner from 5 to 45nM. The cells were maintained in each concentration of paclitaxel for 10 days. Two clones were eventually selected and named "MCF-7^{R}A" and "MCF-7^{R}B" respectively.

For lactate exposure, MCF-7 cells were seeded into 24-well plates (1×10^4/well) and exposed to 0, 5, 10, 20 mM sodium lactate (≥ 99.0%, pH=7.5, Sigma Aldrich, St. Louis, MO, USA) for 12 h after 10 nM paclitaxel exposure for 24h. Then, these cells were washed and harvested for further examination. The study was carried out in accordance with the Helsinki Declaration and approved by the ethics committee of The First Affiliated Hospital of Zhengzhou University.

Flow cytometry analysis

MCF-7, MCF-7^{R}A and MCF-7^{R}B cells were seeded into 24-well plates (1×10^4/well) and treated with10 nM paclitaxel for 24 h. Then, these cells were labeled with propidium iodide (PI) and annexin-V for 15 min using an Annexin V-FITC Apoptosis Detection Kit (Vazyme, Nanjing, China) according to the manufacturer's protocol. All experiments were repeated for three times.

Determination of cell viability

To evaluate cell viability, MCF-7, MCF-7^{R}A and MCF-7^{R}B cells were plated into wells (1×10^4/well) of 96-well plates. The culture medium was replaced with fresh medium containing various doses ($0\sim2\times10^3$ nM) of paclitaxel for 72 h. After that, the media were replaced with 180 μL of RPMI-1640 and 20 μL of using the 3-(4,5-dimethylthiazol-2-yl)-2, -diphenyltetrazolium bromide (MTT) and cell viability was detected following the manufacturer's protocol (Beyotime, Wuxi, China).The absorbance was read at 490 nm on a spectrophotometer (Merinton Instrument, Ann Arbor, MI, USA). The IC50 value was determined using GraphPad Prism 5 software. All experiments were repeated for three times.

SiRNA transfection

Knockdown of PFKFB3 expression was carried out using small interfering RNAs (siRNAs) including scramble siRNA and PFKFB3 siRNA (Santa Cruz Biotech, Santa Cruz, CA, USA). MCF-7^{R}A and MCF-7^{R}B cells were transfected with scramble siRNA or PFKFB3 siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At 48 h after transfection, cells were washed, harvested and presented for further analysis.

Plasmids construction and transfection

PFKFB3gene was extracted from MCF-7 cells and then cloned into the pcDNA3.1 vector (Life technology, Shanghai, China) using the EcoR I and Hind III sites. The recombinant plasmids were transformed to competent cells and then extracted using plasmid extraction kit (Tiangen, Beijing, China), before examined by agarose gel electrophoresis and sequence. MCF-7 cells (1×10^4/well) were planted into 24-well plate and pcDNA3.1/PFKFB3 or blank for plasmids were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After the transfection, the expression of PFKFB3 in MCF-7 was then analyzed by Western blot.

ELISA analysis

IL-6 and IL-8 release into the supernatant of MCF-7, MCF-7^{R}A and MCF-7^{R}B cells were determined with a enzyme-linked immunosorbent assay Kit (ELISA; R&D Systems, Minneapolis, MN, USA) in accordance with the manufacturer's instructions. The absorbance was read at 490 nm using a Merinton spectrophotometer (Merinton Instrument). All of the samples were analyzed in duplicate. The standard curve for protein estimation was made by linear regression analysis.

Lactate and F2,6BP measurements

1×10^4 cells were trypsinized and washed twice with PBS before the measurement of total intracellular F2,6BP. The F2,6BP concentration was normalized to total cellular protein as measured by the bicinchoninic acid (BCA) assay. Lactate production from breast cancer cells were measured using a lactate oxidase-based colorimetric assay read at 540 nm according to the manufacturer's instructions (Beyotime, Wuxi, China) and normalized to cell numbers. All experiments were repeated for three times.

Western blot

Total proteins were extracted from MCF-7, MCF-7^{R}A and MCF-7^{R}B cells and then were separated by SDS-PAGE. Western blot analysis was performed according to standard procedures. B-actin was used as internal reference gene for protein quantification. Primary antibodies includinganti-PFKFB3 (1: 1500, Sigma-Aldrich, St. Louis, MO, USA), anti-caspase 3 (1:1000, Beyotime, Wuxi, China), anti-cleavage caspase 3 (1:1000, Beyotime), anti-TLR4 (1:1000, Abcam, Cambridge, UK), anti-MyD88 (1:1500, Santa Cruz Biotech, Santa Cruz, CA, USA), anti-I-κB (1:1500, Santa Cruz Biotech) and anti-β-actin (1:2000, Abcam) were employed for the detection. Goat anti-mouse IgG (1:10 000) was used as the second antibody following with

enhanced chemiluminescence (ECL, Amersham Pharmacia, NJ, USA) detection.

Statistical analysis

Our data were shown by means ± SD. SPSS17.0 software (Chicago, IL, USA) was used for statistical analysis. Difference comparison was carried out using one-way ANOVA analysis following with post-hoc tests. Differences were considered to be significant when $P<0.05$.

Results

PFKFB3 is induced by paclitaxel in resistant breast cancer cells

Warburg effect is a critical property of cancer cells and may be implicated in the development of paclitaxel resistance (12). PFKFB3 is a critical enzyme involved in the alteration of glucose metabolism in cancer cell (23). To assess whether the PFKFB3 expression in breast cancer cells was modulated by paclitaxel, two clones of paclitaxel resistant cell lines (MCF-7^{R}A and MCF-7^{R}B), along with their parent cell line MCF-7, were established and selected for the examination. MCF-7^{R}A and MCF-7^{R}B were developed by the exposure of MCF-7 to increasing concentrations of paclitaxel in the culture medium for at least 4 months. After the exposure, the cellular morphology of the resistant cells turned to be spindle compared with their parent cells (Figure 1A), when they were exposed to 10 nM paclitaxel for 24 h. Moreover, reduction of apoptotic percentages was observed in MCF-7^{R}A and MCF-7^{R}B cells in comparison to MCF-7 (Figure 1B), as determined by flow cytometry. The IC50 concentrations of MCF-7^{R}A (IC50=1307.2 nM) and MCF-7^{R}B cells (1431.5 nM), measured by MTT assay, were also raised compared with MCF-7 cells (29.6 nM) (Figure 1C). In addition, the results of immunoblot showed that upregulation of PFKFB3 as well as downregulation of cleavage caspase 3 were induced in MCF-7^{R}A and MCF-7^{R}B cells compared MCF-7 cells (Figure 1D, 1E). However, the expression of PFKFB4 was slightly enhanced ($P>0.05$) (Figure 1D, 1E). Thus, our data gave a hint that PFKFB3 might be implicated in the induction of paclitaxel resistance.

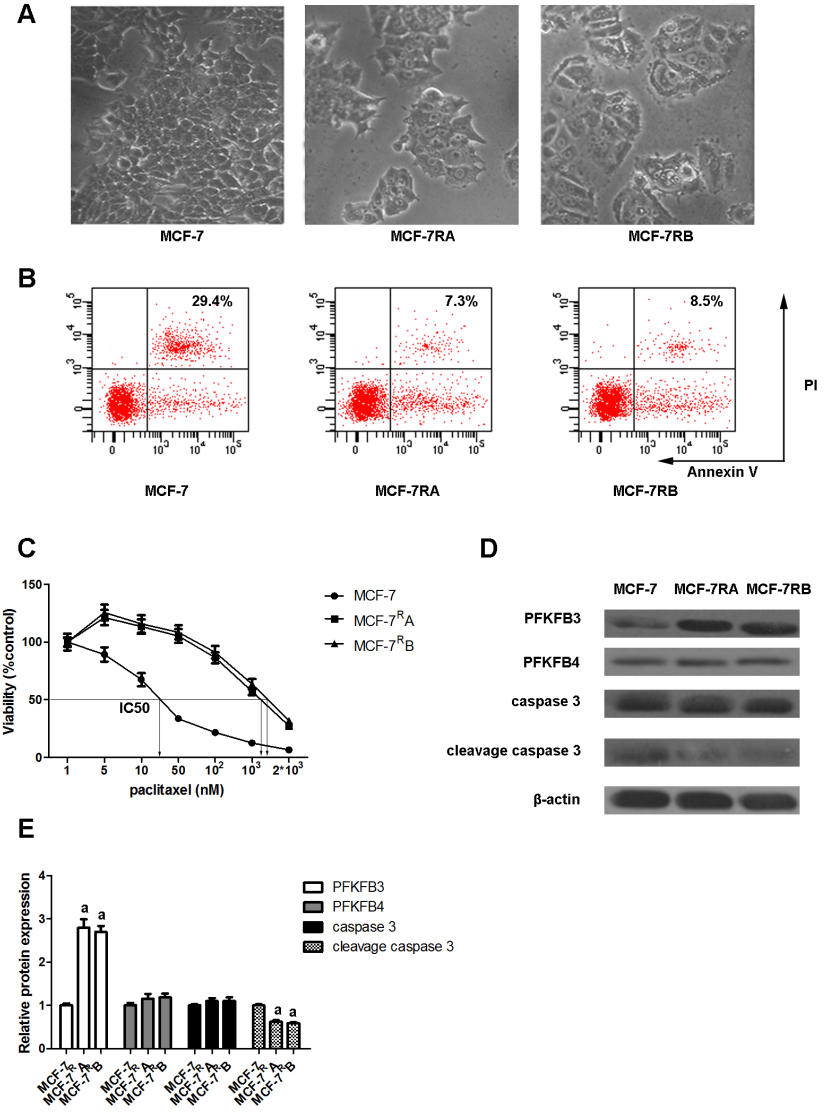

Figure 1. PFKFB3 was overexpressed in two clones of paclitaxel resistant breast cancer cells. MCF-7^{R}A and MCF-7^{R}B were developed by the exposure of MCF-7 to increasing concentrations of paclitaxel for at least 4 months. After that, (A) the morphological alterations of these cells were observed under a microscope (400×); (B) MCF-7, MCF-7^{R}A and MCF-7^{R}B cells were exposed to 10 nM paclitaxel for 24 h and the apoptosis was determined by flow cytometry; (C) IC50 concentrations of MCF-7, MCF-7^{R}A and MCF-7^{R}B cells were measured using MTT method; (D) (E) the expressions of PFKFB3, PFKFB4, caspase 3 and cleavage caspase 3 were analyzed by Western blot and quantified. [a] $p<0.05$, compared with MCF-7.

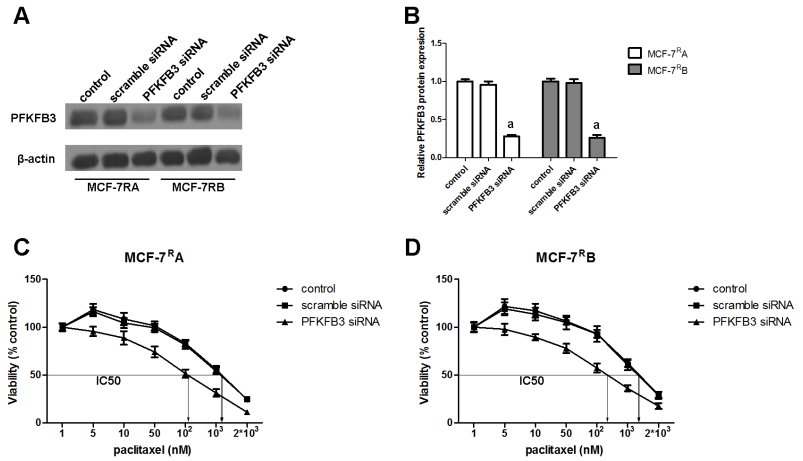

Figure 2. Silencing PFKFB3 expression reduced IC50 concentrations of MCF-7^{R}A and MCF-7^{R}B cells. (A) (B) Scramble or PFKFB3 siRNA was transfected into MCF-7^{R}A and MCF-7^{R}B cells and the expression of PFKFB3 protein was analyzed; (C) (D) IC50 concentrations of MCF-7^{R}A and MCF-7^{R}B cells were measured after scramble or PFKFB3 siRNA transfection. ^{a} $p<0.05$, compared with control.

Silencing PFKFB3 expression enhances paclitaxel sensitivity of breast cancer cells

To further evaluate the role of PFKFB3 in paclitaxel resistance, scramble or PFKFB3 siRNA was transfected into MCF-7^{R}A and MCF-7^{R}B cells to silence PFKFB3 expression. Compared with scramble siRNA transfection, PFKFB3 siRNA significantly inhibited PFKFB3 expression in these cells (Figure 2A, 2B). Furthermore, knockdown of PFKFB3 markedly reduced the IC50 concentrations of MCF-7^{R}A (PFKFB3 siRNA vs control: 161.7 nM vs 1296.4 nM) (Figure 2C) and MCF-7^{R}B cells (PFKFB3 siRNA vs control: 382.6 nM vs 1403.5 nM) (Figure 2D). Our results indicated the requirement of PFKFB3 for paclitaxel resistance.

PFKFB3 contributes toTLR4 signaling activation in breast cancer cells

Evidence has emerged that TLR4 signaling activation contributes to paclitaxel resistance (24). Therefore, we examined the expressions of TLR4 and MyD88 in MCF-7, MCF-7^{R}A and MCF-7^{R}B cells under paclitaxel exposure for 24 h. As it was shown in the figure, the levels of TLR4 and MyD88 protein were significantly higher than those in MCF-7 (Figure 3A, 3B). Subsequently, knockdown of PFKFB3 expression in MCF-7^{R}A and MCF-7^{R}B cells markedly diminished TLR4 and MyD88 expressions (Figure 3C, 3D, 3E, 3F), accompanied by the reduction of IL-6 and IL-8 release (Figure 3G, 3H). The results revealed that PFKFB3 might seduce the activation of TLR4 signaling in breast cancer cells after paclitaxel exposure.

Lactate release stimulated by PFKFB3 is responsible for TLR4 signaling activation and paclitaxel resistance

As PFKFB3 phosphorylates F6P to F2,6BP or dephosphorylates F2,6BP to F6P and may set the intracellular concentration of F2,6BP, which determines the glycolytic rate of cancer cells (17), the glycolysis of MCF-7, MCF-7^{R}A and MCF-7^{R}B cells were then detected. Compared with MCF-7 cells, the production of F2,6BP and lactate in MCF-7^{R}A and MCF-7^{R}B cells was remarkably enhanced compared with that in MCF-7 cells (Figure 4A, 4B). Furthermore, overexpression of PFKFB3 by pcDNA3.1/PFKFB3 vector transfection

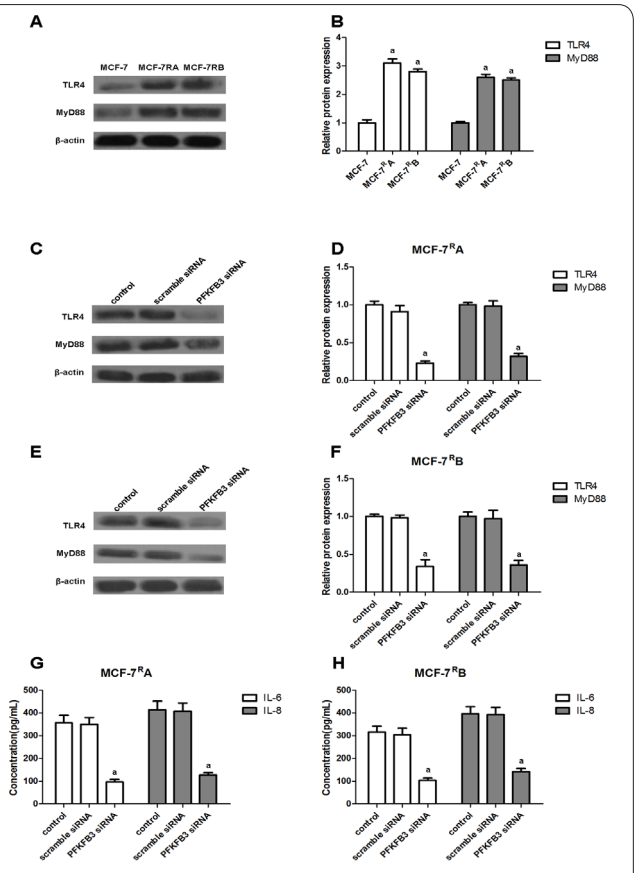

Figure 3. PFKFB3 modulated TLR4 signaling activation in breast cancer cells. (A) (B) The expressions of TLR4 and MyD88 in MCF-7, MCF-7^{R}A and MCF-7^{R}B cells were analyzed; (C) (D) the expressions of TLR4 and MyD88 inMCF-7^{R}A cells were examined after scramble or PFKFB3 siRNA transfection; (E) (F) the expressions of TLR4 and MyD88 in MCF-7^{R}B cells were examined after scramble or PFKFB3 siRNA transfection; (G) (H) IL-6 and IL-8 release was detected in MCF-7^{R}A and MCF-7^{R}B cells; ^{a} $p<0.05$, compared with MCF-7 or control.

promoted F2,6BP and lactate production in MCF-7 cells (Figure 4C, 4D, 4E, 4F). Lactate could stimulate TLR4 signaling activation and the expression of inflammatory genes in human U937 histiocytes (25). We then speculated that the augmented production of lactate might be responsible for TLR4 signaling activation and paclitaxel resistance. MCF-7 cells were incubated with 0, 5, 10, 20 mM lactate for 12 h after paclitaxel exposure. At the end

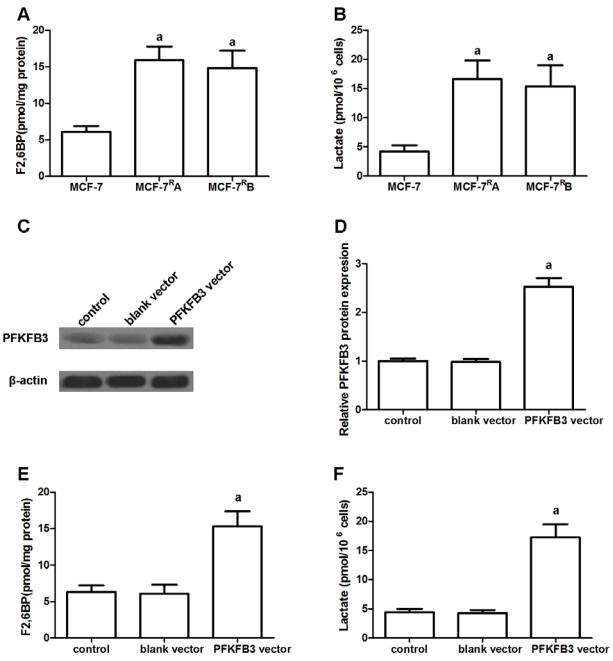

Figure 4. PFKFB3 regulate glycolysis in breast cancer cells. (A) F2, 6BP and (B) lactate production in MCF-7, MCF-7^{R}A and MCF-7^{R}B cells; (C) (D) blank or pcDNA3.1/PFKFB3 vector was transfected into MCF-7 cells and the expression of PFKFB3 was analyzed; (E) F2, 6BP and (F) lactate production in MCF-7 cells were measured after blank or pcDNA3.1/PFKFB3 vector transfection. MCF-7 cells were incubated with 0, 5, 10, 20mM lactate for 12 h after paclitaxel exposure. [a] $p<0.05$, compared with MCF-7 or control.

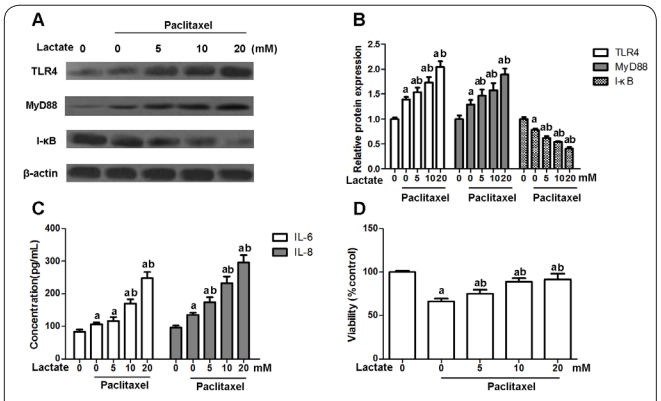

Figure 5. Lactate activated TLR4 signaling and enhanced breast cancer cell viability with paclitaxel exposure. MCF-7 cells were incubated with 0, 5, 10, 20mM lactate for 12 h after paclitaxel exposure. At end of the treatment, (A) (B) TLR4, MyD88 and I-κB expressions were analyzed; (C) IL-6 and IL-8 release was determined; (D) cell viability was detected by MTT. [a] $p<0.05$, compared with 0mM lactate exposure without paclitaxel conditioning; [b] $p<0.05$, compared with 0mM lactate exposure with paclitaxel conditioning.

of the treatment, the expressions of TLR4, MyD88 and I-κB, an inhibitor of NF-κB, were analyzed by immunoblot. Our data showed that lactate upregulated TLR4 and MyD88 expression and downregulated I-κB expression in a dose-dependent manner (Figure 5A, 5B). Furthermore, lactate incubation significantly enhanced IL-6 and IL-8 release as well as cell viability of MCF-7 cells under paclitaxel condition (Figure 5C, 5D).

Discussion

Paclitaxel, an established cytotoxic drug for breast cancer, was reported to obtain response rates varying from 34% to 48% in patients (26-28). However, most of these patients relapse upon or after treatment. Although many efforts have been taken to improve therapeutic strategies, almost all patients will eventually develop tumors that are non-responsive to these strategies (29). Therefore, novel molecular mechanism needs to be understood to combat the development of paclitaxel resistance. In this study, to study paclitaxel resistance in vitro, we established two clones of breast cancer cell lines, MCF-7^{R}A and MCF-7^{R}B in resistance to paclitaxel by a long term of paclitaxel exposure. These cells were characterized by lower apoptotic rate and higher IC50 concentrations in comparison to their parent cells. Interestingly, a raised expression of PFKFB3, a critical enzyme in glycolytic metabolism, was also observed in these paclitaxel-resistant cells, which might suggest the involvement of PFKFB3 in the state of paclitaxel resistance of breast cancer cells. However, the expression of PFKFB4, another essential enzyme which was reported to be overexpressed in breast cancer and involved in tumorigenesis (30-33), was slightly enhanced in MCF-7^{R}A and MCF-7^{R}B cells. Our data also did not exclude its role in paclitaxel resistance as its expression was detected among MCF-7, MCF-7^{R}A and MCF-7^{R}B cells (Figure 1D). In our further investigation, knockdown of PFKFB3 expression significantly reduced IC50 concentrations of MCF-7^{R}A and MCF-7^{R}B. These results might strengthen the point that PFKFB3 was required for paclitaxel resistance in breast cancer cells. In addition, the role of PFKFB3 in cell proliferation and autophagy, which served as a survival strategy under stresses, has been featured by other researchers (34, 35).

Notably, TLR4 activation has recently been reported to play a role in paclitaxel resistance. In breast cancer, compared with TLR4-negative tumors, TLR4-positive tumors obtained enhanced local and systemic inflammation concomitant as well as increased lymphatic metastasis with significantly worse outcome after paclitaxel treatment (36). TLR4 depletion in MDA-MB-231 cells downregulated prosurvival genes, along with decreased IC50 concentration by 2- to 3-fold in response to paclitaxel in vitro and reduced recurrence rate by 6-fold in vivo (37). Moreover, in ovarian tumor cells, TLR4 has a significant function in tumor cell growth as well as their resistance to paclitaxel (38). In the present study, we showed that TLR4 and MyD88 expressions were augmented in MCF-7^{R}A and MCF-7^{R}B cells compared with those in MCF-7 cells. However, when the expression of PFKFB3 were inhibited in MCF-7^{R}A and MCF-7^{R}B cells, these cells obtained lower expressions of TLR4 and MyD88 as well as IL-6 and IL-8 release.

Lactate accumulation was observed in aggressive breast cancer cell line and in the tumor core of human solid tumors (39, 40). Breast cancer cells could tolerate and employ lactate at clinically relevant concentrations in vitro and in vivo (40). Moreover, lactate helps to enhance cancer cell survival by the inhibition of immune response via maintaining a slightly acidic micro-environment (41). Importantly, lactate enhances LPS-stimulated expression of inflammatory genes and

promotes TLR4 activation in macrophages (25, 42). The current study showed that the production of F2,6BP and lactate in MCF-7^{R}A and MCF-7^{R}B cells was higher than that in MCF-7 cells. Meanwhile, overexpression of PFKFB3 in MCF-7 boosted up F2,6BP and lactate production, indicating the promotion of glycolysis in breast cancer cells by PFKFB3. Moreover, when MCF-7 cells were challenged with increasing doses of lactate, the expression of TLR4, IL-6 and IL-8 in these cells as well as cell viability were increased with reduced expression of I-κB. The results indicated the production of lactate stimulated by PFKFB3 might, to some extent, mediated TLR4 signaling activation and caused paclitaxel resistance.

In summary, the current study demonstrated that PFKFB3, induced in paclitaxel resistant breast cancer cells, was indispensable for maintaining the state of paclitaxel resistance. PFKFB3 modulated TLR4 signaling activation via lactate production in breast cancer cells. Our data, though obtained in vitro, might provide novel views for breast cancer therapy in counteracting drug resistance.

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