TRAF6 promotes chemoresistance to paclitaxel of triple negative breast cancer via regulating PKM2-mediated glycolysis

Han Xu (doc_hxu@fudan.edu.cn)  
Jing'an District Center Hospital

Longzhi Li  
Jing'an District Center Hospital

Bing Dong  
Jing'an District Center Hospital

Ji Lu  
Jing'an District Center Hospital

Kun Zhou  
Jing'an District Center Hospital

Xiaoxin Yin  
Jing'an District Center Hospital

Huishen Sun  
Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine

Article

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Abstract

Ample evidence reveals that glycolysis plays an important role in cancer progression; however, the underlying mechanism of its drug resistance is still worth being further explored. TRAF6, a E3 ubiquitin ligase, is well known to overexpress in various types of cancers, which predicts poor prognosis. In our study, we discovered that TRAF6 expressed more significantly in triple negative breast cancer (TNBC) than in other subtypes of breast cancers, promoting chemoresistance to paclitaxel; that the inhibited TRAF6 expression in the chemoresistant TNBC (TNBC-CR) cells enhanced the sensitivity by decreasing glucose uptake and lactate production; that TRAF6 regulated glycolysis and facilitated chemoresistance via binding directly to PKM2; and that overexpressing PKM2 in the TNBC-CR cells with TRAF6 knocked down regain significantly TRAF6-dependent drug resistance and glycolysis. Additionally, we verified that TRAF6 could facilitate PKM2-mediated glycolysis and chemoresistance in the animal models and clinical tumor tissues. Thus, we identified the novel function of TRAF6 to promote glycolysis and chemoresistance in TNBC by regulating PKM2, which could provide a potential therapeutic target for TNBC treatment.

Introduction

In breast cancer, which is the most common malignant tumor in women[1], triple negative breast cancer (TNBC), tested as negative expression of progesterone receptor (PR), estrogen receptor (ER) and epidermal growth factor receptor 2 receptor (HER2) by immunohistochemistry, is easy to relapse and metastasize[2]. Given a lack of effective endocrinotherapy and molecular targeted therapy, the main treatment option is chemotherapy for TNBC[3, 4]. However, TNBC patients usually develop chemotherapy resistance, resulting in a poor prognosis[2, 5]. Paclitaxel, routinely approached to breast cancer, was reported to cause mitosis cease and cell death by stabilizing microtubules and inhibiting their decomposition[6]. Despite of the therapeutic benefits paclitaxel possesses, half of TNBC patients could become resistant to the chemotherapy after 0.5-1 year of treatment[7]. Hence, it is imperative that the mechanism of drug resistance in TNBC be investigated as the key approach to improving the patient's prognosis.

Tumor necrosis factor receptor-associated factor 6 (TRAF6), classified as E3 ubiquitin ligase[8], plays a significant role in the processes of cancer biology by triggering cell signaling pathways, including NF-κB signaling pathway[9], MAPK signaling pathway[10] and Wnt/β-catenin pathway[11]. It has been confirmed recently that TRAF6 is a prognostic biomarker, which is likely to overexpress in such cancers as renal cell carcinoma[12], colorectal cancer[13] and pancreatic cancer[9]. TRAF6 was reported to facilitate the occurrence and development of tumors by affecting cell apoptosis, proliferation, survival, and invasion[14]. All this indicates that since TRAF6 regulation could facilitate the modulation of tumor progression of various types, much importance should be attached to the exploration of the exact impact on TNBC chemoresistance.
Cancer cells are preferred to uptake more glucose than the normal cells to support aerobic glycolysis, even in the presence of abundant oxygen, known as the Warburg effect [15–17]. Accumulating evidence suggests that elevated aerobic glycolysis confers on cancer cells a growth advantage, promoting resistance to chemotherapeutics by providing energy and metabolic intermediates[18–20]. Pyruvate kinase M2 (PKM2) is widely upregulated in cancer cells to be involved in cancer metabolism and tumor growth[21, 22]. The breast cancer cells that express PKM2 tend to show glycolysis adaptation, which facilitates them to maintain metastasis capacity[23]. Additionally, previous studies have indicated that inhibiting PKM2 could increase sensitivity to chemotherapeutics in multiple tumors, such as colorectal cancers[24], bladder cancers[25], and multiple myeloma cells[26]. We thus hypothesized that PKM2 could regulate paclitaxel resistance in TNBC by altering glycolysis.

In our study, we discovered that TRAF6 which overexpressed in TNBC-patients-derived specimens was related to chemoresistance; that glycolysis was measured to be promoted in chemoresistant TNBC cells; that the inhibited expression of TRAF6 enhanced the sensitivity of TNBC to paclitaxel; and that that TRAF6 was capable of binding to PKM2 directly to promote TNBC cells glycolysis, which increased resistance to paclitaxel in vivo and vitro. Therefore, our research can provide a potential prognosis molecule and treatment target for TNBC.

Materials And Methods

Patients and clinical tissue samples

A number of 185 invasive ductal cancer tissue samples of TNBC patients were obtained from Jing’an District Central Hospital of Shanghai, with an approval from the ethics committee and informed consents from the patients for experiment. As indicated in Supplemental Table 1, a summary was made of the detailed clinicopathological features.

Cell culture, lentivirus preparation and cell transfection

From Fudan University Cancer Institute were derived MDA-MB-231HM cells (high lung metastasis, simplified as 231HM cells), and from ATCC were obtained MDA-MB-231 cells (simplified as 231 cells), which all were cultured with Dulbecco’s modified Eagle’s medium (Gibco), supplemented with 10% FBS and 100U/ml penicillin, in the temperature of 37°C and humidified environment of 5% CO₂. The plasmids, which carried TRAF6 shRNA1 (5’-GAGAACACCCAGTCACACA-3’), TRAF6 shRNA2 (5’-GCCACGGGAAATATGTAATAT-3’) and PKM2 full-length cDNA, were transfected into 293T cells, respectively, before cultured with fresh medium after eight hours of transfection. When the supernatant with lentivirus was harvested 48 hours later, the cell debris was removed with a 0.45µm filter. These supernatant viruses were used to infect the corresponding cells to generate TRAF6 knockdown or PKM2 overexpression cells. The cells were then screened with 5µg/ml puromycin for a period of 7–10 days; consequently, the expressions of TRAF6 and PKM2 were detected by western blotting.

Cell viability and cell apoptosis assay
CCK-8 kit was used to detect the cell viability. In each well of 96-well cell culture plates were inoculated 5,000 cells. When adhered, the cells were cultured with different concentrations of paclitaxel diluted with complete medium. Forty-eight hours later, the paclitaxel solution was sucked up and the dead cells were cleaned with PBS, followed by an addition of 100µL 5% CCK-8 to each well, which was prepared by medium containing 10% FBS, before incubated in the incubator for 2 hours. Into the 96-well plate was absorbed 95µL liquid so that the OD value was measured at 450nms. According to different OD values of different drug concentrations, the fitting curves were drawn.

The paclitaxel-induced cell apoptosis was analyzed by flow cytometry after the staining of propidium iodide (PI) and Annexin V. 1×10^6 cells were seeded in 60mm dish to be kept for 24 hours and treated with paclitaxel for 12 hours, before trypsinized and washed twice in phosphate-buffered saline (PBS). 1×10^5 cells were resuspended in 100mL binding buffer, to which were added 5mL 2mg/ml Annexin V and 5ml 50mg/ml PI. Incubated in dark for 15 minutes, the cells were detected by flow cytometry.

**qRT-PCR array**

Total RNA was extracted from the cells by FastPure® Cell/Tissue Total RNA Isolation Kit (Vazyme, China) in accordance with the instructions. The reverse transcription of mRNA was performed using QuantiMiR cDNA Kit PrimeScript™ RT Master Mix (TaKaRa, Dalian, China). The RNA level was measured by qRT-PCR using SYBR Premix Ex Taq™ (TaKaRa, Dalian, China), and 18S RNA was used as the endogenous control for mRNA. Fold change was calculated by relative quantification (2-ΔΔCt).

**Western blotting**

RIPA lysis was used to extract total protein, which was to be quantified with BCA protein assay kits (Beyotime, Shanghai, China), and separated by SDS-PAGE gel. The protein was transferred to PVDF membrane, before blocked with quick blocking buffer (Beyotime, Shanghai, China), followed by incubation with specific rabbit antibodies (1:1000, Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. Goat-anti-rabbit secondary antibody conjugated to the horseradish peroxidase (1:1000, Cell Signaling Technology), was incubated at room temperature for 1 hour. Consequently, the proteins were shown with a chemiluminescent horseradish peroxidase substrate (Beyotime, Shanghai, China) to be imaged with an E-Gel Imager (Bio-Rad, Hercules, CA, USA).

**Immunohistochemistry (IHC) assay**

The tissue slides were deparaffinized in xylene and rehydrated in a graded series of ethanol, the sections subjected to antigen retrieval via boiling in 0.01 mol/L sodium citrate buffer (pH 6.0) in a microwave oven for 10 minutes. With the blocking of the endogenous peroxidase activity with 0.3% hydrogen peroxide and of nonspecific protein binding with 1.5% normal goat serum, the sections were incubated overnight with an antibody at 4°C in a humid chamber. After that, the antibodies were localized with the sections incubated with biotinylated goat anti-mouse or goat anti-rabbit IgG for 30 minutes, which were detected with the LSAB system (Dako).

**RNA-sequencing**
Total RNA was isolated from the breast cancer cells to be treated with mRNA Capture Beads (Vazyme) so that polyA RNA was enriched. RNA library was prepared using VAHTS mRNA-seq v2 Library Prep Kit from Illumina (Vazyme), and the paired-end sequencing was performed with Illumina HiSeq 3000 at RiboBio Co., Ltd. To perform the computational analysis of the RNA sequencing data, sequencing reads were compared using the spliced-reads comparator of HISAT2, based on the human genome collection as a reference genome. The gene expression level of each transcript was estimated as reads per million exon model per kilobase. Gene Set Enrichment Analysis (GSEA) was applied to the gene function annotation. When the gene expression multiple changes > 2 and P value < 0.05 through Cufflinks calculation, the genes were considered to be expressed differentially and significantly.

Immunoprecipitation and mass spectrometry analysis

RIPA lysis was used to extract total protein. immunoprecipitated magnetic beads were co-incubated with TRAF6/IgG antibody for 30 minutes, and TRAF6/IgG antibody combined with magnetic beads was co-incubated with the medium for 1 hour at room temperature for antigen precipitation reaction. In the cells, consequently, TRAF6 protein was enriched, and the complex was washed on the magnetic rack. Finally, the protein complexes were eluted from the magnetic beads by heating for 8 minutes in a metal bath at 95 °C. SDS-PAGE was applied to separating protein complex, which was analyzed by liquid chromatography/mass spectrometry (LC/MS) or western blotting. The mass spectrometry was performed at Servicebio Company (Shanghai, China).

Glycolysis assays: glucose uptake, lactate release, ATP production and extracellular acidification rate

The glucose uptake rate was measured with Glucose Assay Kit (BioVision); the ATP levels, detected with ATP Assay Kit (Promega); and the extracellular lactate production, examined with Lactate Assay Kit (BioVision). All the operations were performed according to the instruction manual. The measured values were normalized to the protein concentration. ECAR was detected using the Seahorse XF Glycolysis Stress Test Kit (Agilent Technologies) according to the manufacturer's instructions; the extracellular acidification rate (ECAR), measured under the basal condition, followed by the sequential addition to each well of glucose (10mM), oligomycin (2mM), and 2-deoxyglucose (100mM); and the ECAR values, calculated after normalization cell count, plotted as average ± SD.

Tissue immunofluorescence technique

The paraffin sections were deparaffinized in xylene and rehydrated in a graded series of ethanol, then washed thrice each 5 minutes with 0.01M PBST (PBS containing 0.2% Tween-20). Followingly, the tissue sections underwent antigen retrieval in 0.01 mol/L sodium citrate buffer (pH 6.0) in a water bath kettle at 95°C for 15 minutes. Sealed with 5% donkey serum prepared by PBS for 30 minutes and then cleaned with PBS, the sections were added with 100μL primary antibody (all the primary antibodies administered at 1:1000 dilutions by PBS) for incubation overnight at 4 °C. Afterwards, the sections were washed thrice with PBST, before added with 100μL fluorescent secondary antibody (all the secondary antibodies at 1:250 dilutions by PBS), for a one-hour incubation at room temperature and away from light. After that,
the sections were cleaned with PBST thrice, before sealed as in the same case of fluorescence in situ hybridization. The protein co-localization was observed under the confocal laser microscopy.

**Tumor xenograft assay**

The female nude mice were obtained from SLRC Laboratory Animal Center (Shanghai, China). The cells of MDA-MB-231, MDA-MB-231-CR, MDA-MB-231-CR/TRA6i and MDA-MB-231-CR/TRA6i/PKM2 were suspended in PBS, from which 10uL cell suspension was respectively added into 90µL PBS to ensure that 1×10⁷ cells were injected into each nude mouse at an injection volume of 100µL.

Three weeks later, paclitaxel was administered through a tail intravenous injection. In the mice, the tumor size was measured and the body weight was weighed weekly. Approximately, the mice were reared for 7 weeks until euthanized, whose tumors were removed to be measured in size and weight. The tumor samples were treated according to the experimental needs.

**Statistical analysis**

The results were expressed as the group Mean ± SEM on GraphPad Prism 8 software. For two-group comparisons, student T-test was applied to the analysis of the data, with the difference considered with statistically significance as P < 0.05. Each experiment was repeated thrice.

**Results**

**Overexpressed TRAF6 to be related with prognosis in TNBC**

As manifested in the immunohistochemistry, TRAF6 was detected to present overexpression in breast cancer (Fig. 1A). Based on qRT-PCR, TRAF6 mRNA level was verified to be higher in breast cancer than in the benign tumor (Fig. 1B). From the analysis of TRAF6 expressions in different subtypes of breast cancer tissues, moreover, TRAF6 mRNA and protein levels were both found to be higher in TNBC than in hormone receptor positive (HR+) and human epidermal growth factor receptor 2 overexpressed (HER2+) breast cancers (Fig. 1C & Fig. 1D). Since TNBC is known to possess a high rate of invasiveness and recurrence, we hypothesized that TRAF6 could have the potential of promoting breast cancer progression.

In the immunohistochemical staining, which was performed in a tissue microarray (TMA) consisting of 185 TNBC specimens to reveal the correlation between TRAF6 expression and TNBC patient’s prognosis, the analysis of Kaplan-Meier curves showed that the high expression levels of TRAF6 predicted lower disease-free survival (DFS) rate (Fig. 1E) and lower overall survival (OS) rate (Fig. 1F).

**TRAF6-promoted paclitaxel resistance in TNBC**

In the research on the association of TRAF6 expression with TNBC chemoresistance, we detected the expression in the needle biopsy specimens of TNBC patients who had received the neoadjuvant chemotherapy. The data showed that TRAF6 protein levels were significantly higher in the TNBC tissues resistant to chemotherapy (TNBC-CS) than in those sensitive to chemotherapy (TNBC-CR) by
immunohistochemistry (Fig. 2A & Fig. 2B). Since paclitaxel is one of prime chemotherapeutics for TNBC, in the calculation of paclitaxel IC50 to investigate the association of TRAF6 with paclitaxel resistance of TNBC in MDA-MB-231 parental cells, MDA-MB-231 chemoresistant cells, MDA-MB-231HM parental cells and MDA-MB-231HM chemoresistant cells, respectively (Fig. 2C), the flow cytometry analysis showed that apoptosis ratio was significantly decreased in the chemoresistant cells when compared with the parental ones (Fig. 2D). As indicated by western blotting, TRAF6 expression was detected to be significantly higher in the chemoresistant cells than in the parental ones (Fig. 2E).

According to the investigation of the biological function of TRAF6, in which two shRNAs were constructed to silence TRAF6 expression, the effect of shRNAs was confirmed by western blotting (Fig. 2F). When downregulated, TRAF6 level decreased significantly the IC50 of paclitaxel in the chemoresistant cells after the drug treatment (Fig. 2G). As indicated by the flow cytometry analysis, TRAF6 expression, when inhibited, increased insignificantly the apoptosis ratio under the treatment of paclitaxel (Fig. 2H). All this indicated that TRAF6 promoted the chemoresistance of TNBC cells to paclitaxel.

**TRAF6-promoted paclitaxel resistance in TNBC cells via glycolysis enhanced**

To further explore the mechanism of chemoresistance in TNBC, an analysis was made of the gene expression differences between TNBC parental cells (MDA-MB-231) and chemoresistant TNBC cells (MDA-MB-231-CR) based on RNA-sequencing; the gene set enrichment analysis (GSEA) indicated that glycolysis metabolic pathway was overactivated in MDA-MB-231-CR cells when compared with MDA-MB-231 parental ones (Fig. 3A). When 2-Deoxy-D-glucose (2-DG) was used to inhibit the glycolysis, the IC50 of paclitaxel was significantly decreased in TNBC-CR cells (Fig. 3B), and the cell apoptosis rate was significantly increased after the treatment of paclitaxel (Fig. 3C). These findings demonstrated that the chemoresistance of TNBC was positively correlated with the activity of glycolysis.

With the changes compared of glucose metabolism activation in MDA-MB-231-CR cells after TRAF6 knockdown by RNA-sequencing to verify whether TRAF6-induced drug resistance was correlated with glycolysis, GSEA showed that the inhibited TRAF6 expression suppressed glycolysis pathway (Fig. 3D), which indicated that the downregulated TRAF6 levels reduced the glucose uptake (Fig. 3E), ATP production (Fig. 3F) and lactate generation (Fig. 3G). Additionally, the extracellular acidification rate (ECAR) assay illustrated that the silenced TRAF6 expression restrained the activity of glycolysis in TNBC-CR cells (Fig. 3H & 3I). These findings revealed that TRAF6 played a promoting role in the glycolysis-mediated drug resistance of TNBC cells.

**Glycolysis-mediated chemoresistance promoted by TRAF6 binding to PKM2**

In the further exploration of the molecular mechanism of TRAF6 regulating glycolysis, qRT-PCR was performed to detect mRNA expression levels of the key glycolysis-related enzymes such as ALDOA, ALDOB, GLUT1, PGK1, PKM1, PKM2, ENO1, G6P, SIRT1 and SIRT4, the results of which showed that
PKM2 was the most significantly upregulated (Fig. 4A). Then the immunoprecipitation technique was employed to determine the interacting proteins (Fig. 4B), before TRAF6 complex was analyzed by liquid chromatography mass spectrometry (LC/MS), in which 21 binding proteins were identified, one of them being PKM2 (Fig. 4C & Supplemental Fig. 1A). Co-immunoprecipitation and western blotting further confirmed the interaction of TRAF6 and PKM2 (Fig. 4D). These findings indicated that TRAF6 regulated the glycolysis pathway by binding directly to PKM2.

In the rescue assays to transduce PKM2 cDNA into TRAF6-downregulating MDA-MB-231-CR and MDA-MB-231HM-CR cells, which was performed to verify our hypothesis that TRAF6 could promote chemoresistance through PKM2, we found that the upregulated PKM2 levels significantly increased the IC50 of paclitaxel (Supplemental Fig. 2A) and decreased the cell apoptosis rate (Supplemental Fig. 2B). Of note was the glucose uptake (Supplemental Fig. 2C), lactate production (Supplemental Fig. 2D) and extracellular acidification rate (ECAR) (Supplemental Fig. 2E & 2F) which were significantly elevated after the introduction of PKM2, respectively.

Furthermore, the western blotting analysis showed that the inhibited TRAF6 expression in TNBC-CR cells lowered the levels of PKM2 and anti-apoptosis proteins of Bcl-2 and Xiap, but increased the expression of pro-apoptosis proteins of Bad and Bak (Fig. 4E). However, overexpressed PKM2 in TRAF6 down-regulated MDA-MB-231-CR and MDA-MB-231HM-CR cells, the levels of apoptosis-related proteins were reversed (Fig. 4E). Furthermore, a significant decrease was observed in the level of phosphorylated STAT3 when the TRAF6 expression was inhibited, while there was a significant increase there when PKM2 was overexpressed (Fig. 4E). Nevertheless, the total STAT3 expression was not altered significantly (Fig. 4E). This indicated that TRAF6 promoted the drug resistance of glycolysis-mediated TNBC cells by binding to PKM2 and phosphorylating STAT3.

**TRAF6-facilitated chemoresistance through PKM2 in vivo**

With the establishment of the xenograft models of MDA-MB-231, MDA-MB-231-CR, MDA-MB-231-CR/TRAf6i and MDA-MB-231-CR/TRAf6i/PKM2 cells to confirm the effect of TRAF6 on TNBC chemoresistance in vivo, in which the mice were treated with paclitaxel once a week three weeks later after inoculation (Fig. 5A). The results revealed a significant decrease or increase in tumor volume (Fig. 5A &B) and weight (Fig. 5C) following the treatment of paclitaxel, when TRAF6 expression was suppressed or PKM2 expression was upregulated. This indicated that the inhibited expression of TRAF6 enhanced the sensibility of TNBC to paclitaxel in vivo, while the upregulated expression of PKM2 facilitated the regaining of the drug resistance to paclitaxel. Therefore, TRAF6 harbored the ability to promote chemoresistance through PKM2 in TNBC (Fig. 5A-5C).

From the application of qRT-PCR technique, it was found that the expressions of PKM2, GLUT1 and Bcl-2 mRNA increased significantly, and that the mRNA expressions of Bad decreased significantly in MDA-MB-231-CR group when compared with the control group (Fig. 5D). However, the inhibited TRAF6 expression lowered the levels of PKM2, GLUT1 and Bcl-2 mRNA and elevated those of Bad mRNA in the TNBC animals, while the upregulated PKM2 expression showed a reversed alteration (Fig. 5D).
Immunohistochemical assays also revealed that the alterations of the protein level were consistent with those of the mRNA level in all groups (Fig. 5E). All this indicated that TRAF6 had the ability of promoting TNBC chemoresistance through PKM2 in vivo.

**TRAF6-promoted TNBC chemoresistance through PKM2-mediated glycolysis in the clinical tumor tissues**

Based on the immunohistochemical analysis of 61 chemoresistant (recurrence/metastasis within three post-operational years) and 124 chemosensitive specimens (non-recurrence/metastasis within three post-operational years) from the TNBC patients to examine the clinical correlation of TRAF6 with chemosensitivity, the results revealed that TRAF6 expression was significantly higher in the chemoresistant than in the chemosensitive TNBC tissues, which indicated a positive correlation of TRAF6 expression with PKM2, GLUT1 and Bcl-2 expression, and a negative correlation of TRAF6 expression with Bad expression (Fig. 6A & B), as shown by the statistic analysis which involved all tumor tissues (Fig. 6C). Tissue immunofluorescence assay also confirmed that TRAF6 was correlated with the expressions of PKM2 and Bcl-2 in chemoresistant TNBC patients, which shared the same location in TNBC tumor tissues (Fig. 6D & E). These findings indicated that TRAF6 promoted drug resistance by regulating PKM2 in the TNBC patients.

**Discussion**

Previous studies have shown that TRAF6 is overexpressed in breast cancer[27], especially in TNBC patients who produce higher expression than those who carry HR+ and HER2+ breast cancer[28]. Moreover, TRAF6 has been reported to facilitate tumor proliferation, invasion and metastasis in multiple types of cancer[27, 29, 30]. All this suggests that TRAF6 can play a vital role in the development and progression of TNBC. Although TRAF6 is involved in various axes of regulation to promote tumors chemoresistance[31–33], there has been a dearth of literature on its role TNBC chemoresistance. In this study, we confirmed that TRAF6 was upregulated in TNBC and discovered the downregulation of TRAF6 which could increase the sensitivity of TNBC cells to paclitaxel.

A previously reported study revealed that TRAF6 regulated PI3K/AKT signaling pathway, leading to the phosphorylation and ubiquitination of AKT and promoting tumor cell growth and proliferation[34]. MAPKs were also reported to be classical signaling pathways mediated by TRAF6 in tumor progression, ultimately resulting in the activation of NF-κB and AP-1[10, 35]; however, whether TRAF6 performs some other regulatory mechanisms that can contribute to chemoresistance is still worth being explored. Evidence showed that elevated glycolysis was conducted in chemoresistant cancer cells to maintain a higher energy requirement as a result of mitochondrial defects[36]. Thus we hypothesized that TRAF6-promoted drug resistance could be correlated with enhanced glycolysis activation. Here in the current study, we discovered that glycolysis pathway was activated in TNBC-CR cells; that 2-DG-inhibited glycolysis increased drug sensitivity; and that downregulated TRAF6 expression significantly facilitated
chemotherapeutic effect with the level of glycolysis decreased. Therefore, we concluded that TRAF6 could promote chemoresistance of TNBC via regulating glycolysis.

Furthermore, we elucidated the specific mechanism of TRAF6 to regulate glycolysis. As a pivotal enzyme in glucose metabolism, PKM2 catalyzes the final rate-limiting step of glycolysis, converting phosphoenolpyruvate and ADP to pyruvate and ATP[37]. Mounting evidence indicates that inhibiting PKM2 expression could delay tumor progression[22, 38, 39], and also could increase sensitivity to chemotherapy[40–42]. In our study, we verified that TRAF6 contributed to chemoresistance in TNBC by binding directly to PKM2; that targeting TRAF6 with shRNA downregulated the level of PKM2, enhancing the sensitivity of TNBC to chemotherapeutics in vitro and vivo; and that PKM2 overexpression rescued drug resistance and glycolysis suppressed by TRAF6 depletion in TNBC cells. These findings suggested that PKM2 could serve as a key molecule of TRAF6 to promote TNBC resistant to chemotherapy.

Additionally, we proved that the inhibited TRAF6 expression lowered the expression of anti-apoptosis proteins, promoting the expression of pro-apoptosis proteins, with the phosphorylated level of STAT3 decreased; and that upregulated PKM2 expression significantly increased the level of phosphorylated STAT3 and anti-apoptosis proteins, but reduced the expression of pro-apoptosis proteins, as manifested by the multiple mechanism researches which revealed that PKM2 could translocate into nuclear to activate STAT3[43, 44]. In view of this, we concluded that TRAF6 could be capable of promoting TNBC chemoresistance by PKM2 to activate STAT3 signal pathway. When TARF6 interacts with PKM2, however, the mechanism of PKM2 to be translocated into the nucleus remains unclear.

In summary, in the current investigation on the function of TRAF6 in TNBC chemoresistance and glucose metabolism we identified a brand-new cancer-driving role of TRAF6/PKM2/STAT3 axis, based on the evidence that TRAF6 interacted with PKM2 to enhance glycolysis and then promote TNBC chemoresistance by activating STAT3 at the level of phosphorylation; and that TRAF6 boosted PKM2-mediated drug resistance of TNBC in the xenograft models and clinical tumor tissues. Therefore, our findings uncovered the underlying mechanism of TRAF6 to facilitate chemoresistance in TNBC, which can provide a promising therapeutic target for cancer treatment.

Declarations

Acknowledgements

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Competing interests

The authors declare no competing interests.
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Figures
Upregulated TRAF6 in TNBC to be correlated with poor prognosis

A. TRAF6 expression detected by immunohistochemistry in breast benign tumor and breast cancer, respectively; B. TRAF6 mRNA level detected by qRT-PCR in breast benign tumors and breast cancers, respectively; C. TRAF6 expression detected by immunohistochemistry in hormone receptor positive (HR+),
human epidermal growth factor receptor 2 overexpressed (HER2+) and triple negative breast cancer (TNBC), respectively; D. TRAF6 mRNA level detected by qRT-PCR in HR+, HER2+ and TNBC, respectively; E. Kaplan-Meier analysis showing low TRAF6 expression to predict favorable disease-free survival (DFS) in TNBC patients; F. Kaplan-Meier analysis showing low TRAF6 expression to predict favorable overall survival (OS) in breast cancer patients among TNBC patients; *P < 0.05, **P < 0.01

Figure 2

TRAF6-promoted drug resistance of TNBC cells
A. TRAF6 expressions detected by immunohistochemistry, respectively, in TNBC which were sensitive (TNBC-CS) or resistant (TNBC-CR) to neoadjuvant chemotherapy; B. Quantitative analysis of TRAF6 expression levels in TNBC-CS and TNBC-CR tissues; C. IC50 values of paclitaxel calculated in TNBC parental cells and chemoresistant cells by CCK-8; D. Cell apoptosis rate analyzed by flow cytometry in TNBC parental and chemoresistant cells; E. Western blotting used to detect the expression of TRAF6 in TNBC parental and chemoresistant cells; F. Two types of shRNAs introduced to suppress TRAF6 expression, which was confirmed by Western blotting; G. IC50 values of paclitaxel measured in TNBC-CR cells by CCK-8 with TRAF6 downregulated; H. Paclitaxel-induced cell apoptosis ratio analyzed by flow cytometry in TNBC-CR cells with TRAF6 expression inhibited; **P < 0.01
Figure 3

TRAF6-enhanced glycolysis to facilitate TNBC chemoresistance

A. RNA-sequencing and Gene Set Enrichment Analysis showing that glycolysis pathway was activated in TNBC CR cells; B. IC50 values of paclitaxel calculated by CCK-8 in TNBC-CR cells with glycolysis inhibited with 2-DG; C. Paclitaxel-induced cell apoptosis ratio examined by flow cytometry in TNBC-CR cells with
glycolysis inhibited with 2-DG; D. RNA-sequencing and Gene Set Enrichment Analysis indicating that glycolysis pathway was suppressed with TRAF6 expression downregulated; E-G. Glucose uptake, ATP production, and lactate production measured in TNBC CR cells with TRAF6 downregulated; H-I. Extra cellular acidification rate(ECAR) monitored in TNBC-CR cells with TRAF6 expression inhibited; **P < 0.01

**Figure 4**

**A**

![Figure A](image)

**B**

![Figure B](image)

**C**

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**D**

![Figure D](image)

**E**

![Figure E](image)
The interaction of TRAF6 with PKM2 in TNBC cells

A. qRT-PCR-based heatmap showing significant difference in the expression of glycolysis-related genes between TNBC parental (MDA-MB-231) and chemoresistant cells; B. The binding proteins of TRAF6 identified by immunoprecipitation and silver nitrate staining; C. TRAF6 binding proteins analyzed by mass spectrometry (MS); D. Co-IP and western blotting used to testify the interaction between TRAF6 and PKM2; E. The expression of the related proteins in TNBC-CR cells detected by western blotting with TRAF6 downregulated or PKM2 overexpressed
Figure 5

Downregulated TRAF6 to enhance the efficacy of paclitaxel by inhibiting glycolysis in vivo

A. Tumor growth of the animals injected with 231, 231-CR, 231-CR/TRAFl6i and 231-CR/TRAFl6i/PKM2 cells to be treated with paclitaxel three weeks later; B. Representative images of the tumors dissected; C. The weight of the tumors analyzed in all groups; D. Relative mRNA levels of glycolysis and apoptosis
detected by qRT-PCR in the related genes; E. The expression of glycolysis and apoptosis detected by immunohistochemistry in the related proteins of the tumor tissues; *P < 0.05, **P < 0.01

Figure 6

TRAF6-promoted glycolysis and chemoresistance in the clinical tissues of TNBC
A. Immunohistochemistry assays of TRAF6, PKM2, GLUT1, Bcl-2 and Bad expression shown in TNBC-CR and TNBC-CS tumor tissues; B. Statistically expressive ratio of TRAF6, PKM2, GLUT1, Bcl-2 and Bad in TNBC-CR and TNBC-CS tumor tissues; C. Correlation analysis of TRAF6 expression with PKM2, GLUT1, Bcl-2 and Bad expression in TNBC tissues; D. TRAF6 and PKM2 expression analyzed by immunofluorescence assay in TNBC-CR and TNBC-CS tumor tissues; E. TRAF6 and Bcl-2 expression analyzed by immunofluorescence assay in TNBC-CR and TNBC-CS tumor tissues; **P < 0.01

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- SFigure1.jpg
- SFigure2.jpg
- SupplementalTable1.docx