Regulatory role of tumor necrosis factor receptor-associated factor 6 in breast cancer by activating the protein kinase B/glycogen synthase kinase 3β signaling pathway

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Abstract. Tumor necrosis factor receptor-associated factor 6 (TRAF6) is an endogenous adaptor of innate and adaptive immune responses, and serves a crucial role in tumor necrosis factor receptor and toll-like/interleukin-1 receptor signaling. Although studies have demonstrated that TRAF6 has oncogenic activity, its potential contributions to breast cancer in human remains largely uninvestigated. The present study examined the expression levels and function of TRAF6 in breast carcinoma (n=32) and adjacent healthy (n=25) tissue samples. Compared with adjacent healthy tissues, TRAF6 protein expression levels were significantly upregulated in breast cancer tissues. Reverse transcription-quantitative polymerase chain reaction analysis revealed a significant upregulation of the cellular proliferative marker Ki-67 and proliferation cell nuclear antigen expression levels in breast carcinoma specimens. Furthermore, protein expression levels of the accessory molecule, transforming growth factor β-activated kinase 1 (TAK1), were significantly increased in breast cancer patients, as detected by western blot analysis. As determined by MTT assay, TRAF6 exerted profoundly proliferative effects in the MCF-7 breast cancer cell line; however, these detrimental effects were ameliorated by TAK1 inhibition. Notably, protein kinase B (AKT)/glycogen synthase kinase (GSK)3β phosphorylation levels were markedly upregulated in breast cancer samples, compared with adjacent healthy tissues. In conclusion, an altered TRAF6-TAK1 axis and its corresponding downstream AKT/GSK3β signaling molecules may contribute to breast cancer progression. Therefore, TRAF6 may represent a potential therapeutic target for the treatment of breast cancer.

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Introduction

To date, breast cancer is the most common female malignancy diagnosed, and the most frequent cause of cancer incidence rate among women worldwide (1). Despite advances in diagnosis oncology, the early detection and treatment of breast cancer remains a significant problem due to its metastatic nature (2). Preventing breast cancer prior to its development may be the most effective way to reduce mortality resulting from this disease (3). Previous research has emphasized that breast cancer is a highly heterogeneous group of diseases that differ in their prognosis and response to treatment. The development of breast cancer is thought to occur via a multi-stage process, and a large number of molecules are considered to be involved in the tumorigenesis and progression (4). Therefore, it is critical to investigate the mechanisms underlying breast tumorigenesis and to develop therapeutic strategies that are effective for the early diagnosis and management of breast cancer.

The tumor necrosis factor receptor associated factor (TRAF) protein family were initially identified as adaptor proteins that couple tumor necrosis factor receptor family members to signaling pathways, and are signal transducers of toll/interleukin-1 receptor family members (5). All TRAF proteins share a C-terminal homology region, termed the TRAF domain, that is able to bind to the cytoplasmic domain of receptors, and to other TRAF proteins. Previous studies have confirmed that TRAF proteins are important regulators of cell death and cellular responses to stress (6). To date, seven

members of the TRAF family have been identified, which are termed TRAF1-7 (7). Notably, TRAF6 is a unique adaptor protein of the TRAF family that consists of 530 amino acids, including a highly conserved TRAF6 domain at the C terminus, and an activated coiled-coil domain at the N terminus. The interaction of TRAF6 and other ubiquitin conjugating enzymes is required for IκB kinase activation and downstream signaling of the nuclear factor (NF)-κB transcription factor. TRAF6 also interacts with the transforming growth factor β (TGF-β) receptor complex and is required for mitogen activated protein kinase (MAPK) activation. Since TRAF6 may activate several signaling pathways simultaneously, it serves a crucial role in the growth, proliferation, differentiation and death of cells (8). Furthermore, TRAF6 has been identified in humans and mice (9). Upregulation of TRAF6 expression has been detected in glioma, osteosarcoma, esophageal squamous cell carcinoma, and colon and lung cancer tissues (10). However, there have been no further mechanistic studies regarding the contribution of TRAF6 expression to the development of breast cancer. Therefore, the present study aimed to investigate the pathophysiological contribution of TRAF6 expression to breast tumorigenesis in breast cancer patients.

Materials and methods

Tissue specimens. Human breast tissues (n=32) were obtained from Jiangsu Cancer Hospital (Nanjing, China). The patients had not previously received chemotherapy or radiotherapy. Adjacent healthy tissues were used as a control (n=25). All cases were diagnosed by two experienced pathologists. This study was approved by the institutional review board at Jiangsu Cancer Hospital, and signed informed consent was obtained from all patients and their relatives.

Antibodies and reagents. Antibodies against the following proteins were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA): TRAF6 (cat. no. 8028; 1:1,000), phosphorylated (p)-transforming growth factor β-activated kinase 1 (TAK1); cat. no. 4508; 1:1,000), TAK1 (cat. no. 5206; 1:1,000), p-protein kinase B (AKT)^{Ser473} (cat. no. 4060; 1:1,000), AKT (cat. no. 4691; 1:1,000), p-glycogen synthase kinase (GSK)3β (cat. no. 9322; 1:1,000), GSK3β (cat. no. 9315; 1:1,000), and GAPDH (cat. no. 2118; 1:1,000). The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit IgG (cat. no. 7074; 1:2,000; Cell Signaling Technology, Inc.). The Bicinchoninic Acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL, USA). A FluorChem E imager (ProteinSimple; Bio-Techne, Minneapolis, MN, USA) was used for visualization. The TAK1 kinase inhibitor, 5Z-7-Oxozeaenol (5Z-O; cat. no. 3604), was purchased from Tocris Bioscience (Bristol, UK).

Cell culture. The MCF-7 breast cancer cell line was preserved and maintained at 37˚C in high glucose complete Dulbecco's modified Eagle's medium (DMEM; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences) in a humidified 5% CO_2 incubator. The cells were treated with vehicle [0.1% dimethylsulfoxide (DMSO)] or 5Z-O (1,000 nM) combined with 10% FBS in high glucose complete DMEM medium for 24 h, as described previously (11).

Cell viability assay. An MTT assay was conducted to analyze cell viability. Briefly, cells were seeded into 96-well plates at a density of $8x10^3$/well and cultured with 100 µl complete medium (DMEM high glucose containing 10% FBS). After 24 h, 20 µl 5 mg/ml MTT solution (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added into each well. Cells were incubated for 4 h at 37˚C before the culture medium was removed. Following this, 150 µl DMSO (Amresco, LLC, Solon, OH, USA) per well was added and mixed in order to guarantee cytolysis and dissolution of the formazan crystal. The absorbance was measured at a wavelength of 490 nM using a microplate reader (CliniBio 128; ASYS Hitech GmbH, Eugendorf, Austria). Cells treated with 0.1% DMSO instead of the TAK1 inhibitor were used as blank control. Three independent experiments were performed.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was performed as previously described (12). Briefly, total RNA was extracted from frozen human tissue using a TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and reverse-transcribed into cDNA using oligo (dT) primers with a Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science, Penzberg, Germany). PCR amplifications were quantified using SYBR®-Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) and normalized to GAPDH gene expression by the $2^{-\Delta\Delta Cq}$ method (13). The primer sequences used were as follows: Forward, GCAGCCCGTGTA ACTGGAATGA and reverse, GTCTTCTAGAGCCTGGGC CTT for Ki-67; and forward, TGCGGCCGGGTTCAGGAG TCA and reverse, CAGGCAGGCGGGAAGGAGGAAAGT for proliferating cell nuclear antigen (PCNA). Cycling parameters consisted of 95˚C 10 min, followed by 40 cycles at 95˚C for15 sec (denaturing) and 60˚C for 30 sec (annealing and extension). All samples were analyzed in triplicate for each specific gene.

Western blotting. Western blotting was performed as previously described (14). Total proteins extracted from frozen human tissue were first lysed in radioimmunoprecipitation assay lysis buffer (Biouniquer Technology, Beijing, China) on ice for 30 min, followed by centrifugation at 14,000 x g for 15 min at 4˚C to remove cell pellet. Protein concentrations were measured using a BCA Protein Assay kit. Proteins (50 µg) were separated by 8-12% SDS-PAGE (Invitrogen; Thermo Fisher Scientific, Inc.) and transferred onto polyvinylidene difluoridemembranes (Merck KGaA). The membranes were blocked in TBS with Tween-20 containing 5% skimmed milk powder for 1 h at room temperature, and incubated with various primary antibodies overnight at 4˚C. Following this, membranes were incubated with secondary antibodies for 1 h at room temperature, and were visualized with Enhanced Chemiluminescent reagents (cat. no. 170-5061; Bio-Rad Laboratories, Inc., Hercules, CA, USA) using a FluorChem E imager according to the manufacturer's protocol. Protein expression levels were normalized against GAPDH values. All experiments were conducted at least in triplicate.

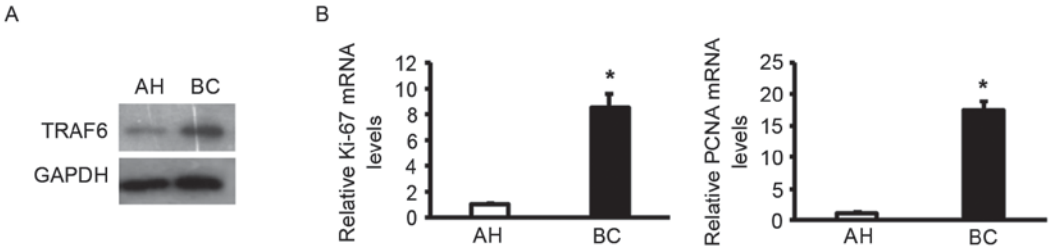

Figure 1. TRAF6 expression is upregulated in human BC. (A) Representative western blot images of TRAF6 protein expression levels from AH (n=25) and BC (n=32) specimens of TRAF6 protein expression levels. GAPDH served as an internal control. (B) Reverse transcription-quantitative polymerase chain reaction of Ki-67 and PCNA in the AN and BC groups. Data are presented as the mean ± standard deviation of three to four independent experiments. *P<0.05 vs. AN. AH, adjacent healthy tissues; BC, breast carcinoma tissues; TRAF6, tumor necrosis factor receptor-associated factor 6; PCNA, proliferating cell nuclear antigen.

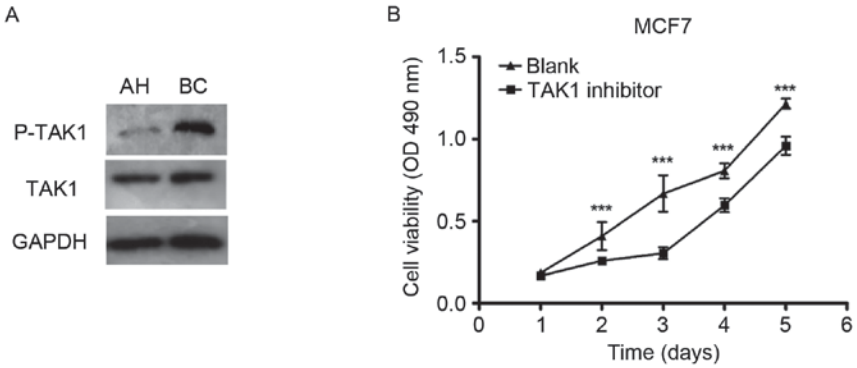

Figure 2. TRAF6 promotes breast carcinoma proliferation in an TAK1-dependent manner. (A) Representative western blot images of protein expression levels of TAK1 in human BC and AH tissues. (B) The MCF-7 breast cancer cell line was treated with TAK1 inhibitor 5Z-7-oxozeaenol (dose: 1,000 nM), and a further MTT assay was performed to determine the activity of proliferation at various time points. Data are presented as the mean ± standard deviation of three to four independent experiments. ***P<0.001 vs. TAK1 inhibit or at the same time point. OD, optical density; TRAF6, tumor necrosis factor receptor-associated factor 6; p, phosphorylated; AH, adjacent healthy tissues; BC, breast carcinoma tissues; TAK1, transforming growth factor β-activated kinase 1.

Statistical analysis. Data are presented as the mean ± standard deviation. For two-group comparisons, Gaussian samples were compared using the two-tailed Student's t-test, while non-Gaussian samples were compared employing the non-parametric Mann-Whitney U test. Statistical analyses were performed with SPSS software version 21.0 (IBM SPSS, Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

TRAF6 expression is upregulated in human breast cancer. Protein expression levels of TRAF6 were determined in breast cancer samples to determine if there was an association. Western blotting revealed that the expression of TRAF6 was higher in breast carcinoma specimens compared with adjacent healthy tissues (Fig. 1A), which implies a role of TRAF6 in tumor progression. RT-qPCR analysis was subsequently performed to determine the association between TRAF6 expression and pathological features. Notably, TRAF6 expression was positively associated with the induction of a series of cellular markers for proliferation at the mRNA level, including Ki-67 and PCNA (Fig. 1B). Collectively, the altered pattern of TRAF6 expression suggested that TRAF6 may be associated with cell proliferation in the development of human breast cancer.

TRAF6 promotes breast carcinoma proliferation in an TAK1-dependent manner. To elucidate the potential molecular mechanisms underlying the effects of TRAF6 on cell proliferation in human breast cancer, the expression levels/activities of TRAF6 signaling molecules (the accessory molecules TAK1) were examined. Protein expression levels of phosphorylated-TAK1 were demonstrated to be upregulated in human breast carcinoma tissues compared with adjacent healthy tissues, as indicated by western blot analysis (Fig. 2A). However, total TAK1 protein expression levels did not differ between the groups (Fig. 2A). The specific TAK1 inhibitor 5Z-O was used to clarify whether the TAK1 signaling pathway is the main molecular event involved in the cell proliferative role of TRAF6 in human breast cancer. 5Z-O significantly impaired the effects of TRAF6 on tumor cell proliferation in MCF-7 cells, as demonstrated by the MTT analysis (Fig. 2B). These results indicated that TAK1 is critical for the TRAF6-induced cell proliferative effect that occurs during breast tumorigenesis.

TRAF6 regulates breast tumorigenesis via activation of the AKT/GSK3β signaling pathways. Increasing evidence has suggested that regulation of the AKT/GSK3β signaling cascade may regulate cellular events critical for tumorigenesis. Activation of the AKT/GSK3β signaling pathway was therefore investigated. The expression levels p-AKT and p-GSK3β were

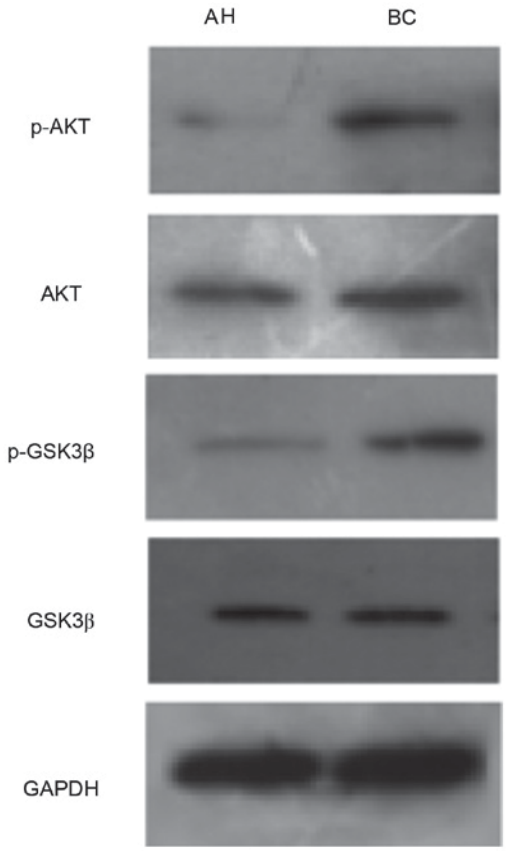

Figure 3. TRAF6 modulates breast tumorigenesis via the activation of the AKT/GSK3β signaling pathway. Representative western blot images of AKT and GSK3β protein expression levels in BC and AH groups. GAPDH served as an internal control. AH, adjacent healthy tissues; BC, breast carcinoma tissues; p, phosphorylated; AKT, protein kinase B; GSK3β, glycogen synthase kinase-3β.

higher in the human breast carcinoma group compared with the adjacent healthy tissues group. However, expression levels of AKT and GSK3β did not differ between the two groups (Fig. 3). These results demonstrated that the proliferative role of TRAF6 may be associated with activation of AKT/GSK3β signaling during breast tumorigenesis.

Discussion

The present study provided evidence of the critical role of the TRAF superfamily member TRAF6 in human breast cancer. Notably, protein expression levels of TRAF6 were observed to be upregulated in human breast carcinoma, compared with adjacent healthy tissues. In addition, the cellular proliferative marker Ki-67 and PCNA mRNA expression levels were significantly upregulated in breast carcinoma tissues compared with adjacent healthy tissues, indicating that TRAF6 expression may be associated with these markers. Mechanistically, it was further demonstrated that TRAF6 may bind to the accessory molecule TAK1, potentially via the AKT/GSK3β signaling pathways, thereby initiating cell proliferation. Therefore, TRAF6 may represent a promising therapeutic target for the treatment of human breast cancer.

TRAF6 may regulate an adverse array of physiological processes, including adaptive and innate immunity, bone metabolism, and the development of several tissues, including lymph nodes, mammary glands, skin and the central nervous system (5). Previous studies have demonstrated that upregulation of TRAF6 occurs in many types of cancer, which suggests that TRAF6 may serve an important role in tumorigenesis and tumor progression (15,16). The present study demonstrated that breast carcinoma specimens had an increase in TRAF6 expression compared with the adjacent healthy tissues, consistent with a study conducted by Bilir *et al* (17) that revealed that serum TRAF6 expression levels were higher in patients with non-metastatic triple-negative breast cancer. The present study demonstrated that mRNA expression levels of cellular markers for proliferation Ki-67 and PCNA were upregulated in breast cancer tissues compared with adjacent healthy tissues, which indicated that TRAF6 may be involved in cell proliferation in human breast tumorigenesis. Peng *et al* (18) reported that TRAF6 induced proliferation of U-87 MG human glioma cells. Furthermore, the proteasome inhibitory effect of bortezomib on TRAF6 was able to restrain multiple myeloma cell proliferation (19). Therefore, further investigation is required to elucidate the underlying mechanisms of TRAF6 in breast tumorigenesis.

Previous studies have demonstrated that the TRAF6-catalyzed Lys-63-linked polyubiquitination of TAK1 at Lys-34 may alter the conformation of TAK1 to function as a central, key regulatory sensor for extra-cellular cues (20,21). In the present study, it was observed that the accessory molecule TAK1 was significantly augmented, suggesting that the TRAF6-mediated regulation of tumor cell proliferation may be directly associated with its downstream target, TAK1. This hypothesis was supported by the findings that a blockade of TAK1 activation induces the TRAF6-elicited cell proliferative response. Such findings are consistent with Xiao *et al* (22), who demonstrated that TRAF6 and its intrinsic ubiquitin E3 ligase activity promotes myogenic differentiation and muscle regeneration via TAK1. Therefore, the proliferative role of TRAF6 in breast tumorigenesis may be dependent, at least partially, on the activation of TAK1. Furthermore, it is well documented that TRAF6-mediated TAK1 activity regulates cell survival, differentiation and inflammatory responses via many diverse signals, including the NF-κB and MAPK cascades (23). To further understand the underlying mechanism responsible for TRAF6/TAK1 signaling, the activation of their downstream pathways was investigated. The results of the present study indicated that activation of AKT and its downstream activator GSK3β was greatly enhanced. Consistent with previous studies, Yoon *et al* (24) also demonstrated that TRAF6 positively regulates cell survival by regulating AKT/GSK3β cascades. In terms of proliferation, previous studies have confirmed that AKT/GSK3β signaling promotes cell cycle progression by upregulating the positive regulators of cell growth including PCNA and cyclin D1 (25-27). Indeed, in the MDA-MB-231 breast cancer cell line, fangchinoline inhibited cell proliferation via suppression of the AKT/GSK3β signaling pathway (28). Furthermore, dihydroartemisinin inhibited cell proliferation via suppression of the AKT/GSK3β signaling pathway in the A549 lung cancer cell line (29). Taken together, these results indicated that AKT/GSK3β signaling pathway is involved in TRAF6-mediated human breast cancer tumorigenesis, but is not the primary molecular mechanism responsible.

However, the potential underlying mechanisms for its involvement in human breast cancer progression remains to be fully elucidated; therefore, other signaling pathways require investigating to further understanding.

In conclusion, the current study provided evidence that TRAF6, a member of the TRAF superfamily, functions as a positive regulator of human breast tumorigenesis by applying the accessory molecule TAK1 to activate the AKT/GSK3β signaling pathway, thereby promoting cell proliferation at the mRNA level. These results implicate TRAF6 as a potential therapeutic target for the prevention and treatment of human breast cancer.

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