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High TRAF6 Expression Is Associated With Esophageal Carcinoma Recurrence and Prompts Cancer Cell Invasion

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Esophageal cancer is one of the most common types of cancer, and it has a poor prognosis. The molecular mechanisms of esophageal cancer progression remain largely unknown. In this study, we aimed to investigate the clinical significance and biological function of tumor necrosis factor receptor-associated factor 6 (TRAF6) in esophageal cancer. Expression of TRAF6 in esophageal cancer was examined, and its correlation with clinicopathological factors and patient prognosis was analyzed. A series of functional and mechanism assays were performed to further investigate the function and underlying mechanisms in esophageal cancer. Expression of TRAF6 was highly elevated in esophageal cancer tissues, and patients with high TRAF6 expression have a significantly shorter survival time than those with low TRAF6 expression. Furthermore, loss-of-function experiments showed that knockdown of TRAF6 significantly reduced the migration and invasion abilities of esophageal cancer cells. Moreover, the pro-oncogenic effects of TRAF6 in esophageal cancer were mediated by the upregulation of AEP and MMP2. Altogether, our data suggest that high expression of TRAF6 is significant for esophageal cancer progression, and TRAF6 indicates poor prognosis in esophageal cancer patients, which might be a novel prognostic biomarker or potential therapeutic target in esophageal cancer.

Key words: Tumor necrosis factor receptor-associated factor 6 (TRAF6); Esophageal cancer (EC); Prognosis; Invasion; Asparaginyl endopeptidase (AEP); Matrix metalloproteinase (MMP)

INTRODUCTION

Esophageal cancer (EC) is one of the most common cancers and has been ranked as the sixth leading cause of cancer mortality worldwide[1–2]. The 5-year overall survival (OS) rate remains below 15%[3–4]. Such poor outcomes in patients with EC are closely associated with diagnosis in advanced stages and the tendency to metastasize early in the disease[5–6]. Therefore, it is very urgent to discover the underlying mechanisms of EC progression, especially metastasis, and identify new biomarkers for EC prognosis and diagnosis.

Attachment of one or more ubiquitin (Ub) monomers, by the ubiquitination enzyme cascade E1, E2, and E3, to cellular proteins has been recognized as a major posttranslational modification that have important functions in various physiological and pathological processes, including cancer development[7–8]. Of these three enzymes, the E3 ligases confer specificity for ubiquitination of substrates[8–9]. Therefore, alteration of Ub ligases is a frequent event in cancer[10]. Tumor necrosis factor receptor-associated factor 6 (TRAF6) is an E3 ligase that mediates the synthesis of Lys63-linked polyubiquitin chains conjugated to proteins, such as IKK, TAK1, and NRIF[11–14]. Amplified TRAF6 locus was found to be a somatic and frequent event in several human cancer types[15]. TRAF6 protein levels are also higher in myelodysplastic syndrome patients[16], further indicating that TRAF6 might be a novel biomarker for cancer diagnosis and prognosis. TRAF6 was suggested as an oncogene for its role in regulating AKT ubiquitination[17]. More recently, TRAF6 was identified as an oncogene in human lung cancer, bridging the RAS and NF-κB pathways[18]. TRAF6 has been found to be a novel protease asparaginyl endopeptidase (AEP) and promotes its protein stability[19]. In EC, TRAF6

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has been found to promote tumorigenesis and metastasis in esophageal squamous cell carcinoma[20–21]. Despite these findings, the relationship between TRAF6 and clinical characteristics, as well as tumor recurrence of EC patients, and the underlying mechanisms in EC have not been well investigated.

Thus, in this study, we aimed to investigate the expression of TRAF6 in a cohort of EC cancerous tissues and the association with clinicopathological factors. Moreover, we constructed two TRAF6 stably knocked-down EC cells and analyzed its migration and invasion abilities. The molecular mechanisms of TRAF6 in mediating EC cell migration and invasion were also studied. Altogether, our results suggest that high expression of TRAF6 increased EC cell migration and invasion. In addition, high TRAF6 expression was significantly related to a short time to relapse in patients suffering from EC, suggesting that TRAF6 might be a novel biomarker or potential therapeutic target in EC treatment.

MATERIALS AND METHODS

Patients and Tissue Samples

Our study was approved by the ethics committee of the Fourth Hospital of Hebei Medical University. Written consent was obtained from patients, or guardians on behalf of minors, enrolled in the study. One hundred forty-six patients with histologically confirmed EC at the Fourth Hospital of Hebei Medical University between January 2005 and December 2013 were recruited for this study. Their diagnoses were independently reviewed by two pathologists, classified by WHO criteria.

Immunohistochemistry

One hundred forty-six blocks of tissue microarray containing EC tissues were constructed using a microarrayer. Serial 4-μm sections were obtained from each block, with the first slide stained with H&E to confirm pathologic diagnosis, and the subsequent slides stained for further immunohistochemistry.

Tissue microarray slides were routinely deparaffinated and rehydrated. The monoclonal antibody against TRAF6 (1:100 dilutions; ab33915; Abcam, Cambridge, MA, USA) was used as the primary antibody. For antigen retrieval, the slides were heated at 98°C in a citrate buffer (pH 9.0) for a total of 20 min and cooled naturally to room temperature. Sections were incubated in 0.3% hydrogen peroxide for 20 min to inactivate endogenous peroxides. The sections were blocked with 5% normal horse serum in PBS for 30 min and then incubated with the primary antibody overnight at 4°C, then stained using a highly sensitive streptavidin–biotin–peroxidase detection system and counterstained with hematoxylin. A negative control was also incorporated using preimmune IgG instead of the primary antibody.

Evaluation of Immunohistochemistry

Two sections per specimen were evaluated by two pathologists independently. Immunoreactive staining was characterized quantitatively according to the percentage of positive cells and staining intensity without prior knowledge of any of the clinicopathological information. We assigned the following proportion scores: 0 if 0% of the tumor cells showed positive staining, 1 if 0% to 10% were stained, 2 if 11% to 50% were stained, 3 if 51% to 75% were stained, and 4 if 75% to 100% were stained. We rated the intensity of staining on a scale of 0 to 3: 0, negative; 1, weak; 2, moderate; and 3, strong. We then combined the proportion and intensity scores to obtain a total score (range 0–12). All patients were designated into negative (score 0), low (score 1–4), moderate (score 5–8), and high (score 9–12) groups based on TRAF6 expression.

Western Blot

To analyze the expression of TRAF6, Western blot assays were performed using the following primary antibodies: rabbit anti-human TRAF6 (ab33915; 1:1,000; Abcam), goat anti-human AEP (af2199; 1:1,000; R&D Systems, Minneapolis, MN, USA), rabbit anti-human MMP2 (ab37150; 1:1,000; Abcam), and mouse anti-actin (1:10,000; Millipore, Billerica, MA, USA). Briefly, cells were lysed with RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.5% Na deoxycholate] containing protease inhibitors (Complete Mini; Roche); 20–30 μg of samples of the lysates was separated on 8%–12% SDS-PAGE gels and transferred to PVDF membranes. The membranes were incubated with primary antibodies overnight at 4°C. The primary antibody incubation was followed by incubation with a HRP-conjugated secondary antibody. The bound antibodies were detected using an ECL kit (PI32209; Pierce).

Lentiviral Vector-Mediated TRAF6 Knockdown

TRAF6 short hairpin RNA (shRNA) sequences were 5′-GGATCTACATTTGGAAGATTG-3′ (TRAF6-KD1) and 5′-GGACCCAAATTATGAGGAAAC-3′ (TRAF6-KD2). After 48 h, the knockdown efficiency was confirmed via quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot.

Lentiviral vectors for human TRAF6-shRNA carrying a green fluorescent protein (GFP) sequence were constructed by Hanyin Co. (Shanghai, P.R. China). The recombinant TRAF6 knockdown lentivirus and the negative control (NC) lentivirus (GFP-lentivirus; Hanyin Co.) were prepared and titered to 10^9 TU (transfection unit)/ml. To obtain the stable TRAF6 knockdown cell line, TE-1 and EC109 cells were seeded in six-well dishes at a density of 2×10^5 cells per well. The cells were then infected with the same titer virus with

8 µg/ml polybrene on the following day. Approximately 72 h after viral infection, GFP expression was confirmed under a fluorescence microscope, and the culture medium was replaced with selection medium containing 4 µg/ml puromycin. The cells were then cultured for at least 14 days. The puromycin-resistant cell clones were isolated, amplified in medium containing 2 µg/ml puromycin for 7 to 9 days, and transferred to a medium without puromycin.

Scratch Assay

Cells were then plated into six-well plates in 200 ml of serum-free RPMI-1640 medium. The assays were conducted in triplicate. The inserts were then placed into the bottom chamber of a 24-well plate containing RPMI-1640 with 10% FBS as a chemoattractant. After 24 h, the top layer of the insert was scrubbed with a sterile cotton swab to remove any remaining cells. The invading cells on the bottom surface were stained with 0.1% crystal violet, examined, counted, and imaged using digital microscopy. The number of cells in five random fields of each chamber was counted, and an average number of cells were calculated.

Matrigel Transwell Assay

Cells were then plated in the top chamber of Transwell assay inserts (Millipore) with a Matrigel-coated membrane containing 8-µm pores in 200 ml of serum-free RPMI-1640 medium. The assays were conducted in triplicate. The inserts were then placed into the bottom chamber of a 24-well plate containing RPMI-1640 with 10% FBS as a chemoattractant. After 24 h, the top layer of the insert was scrubbed with a sterile cotton swab to remove any remaining cells. The invading cells on the bottom surface were stained with 0.1% crystal violet, examined, counted, and imaged using digital microscopy. The number of cells in five random fields of each chamber was counted, and an average number of cells were calculated.

Statistical Analysis

Survival was calculated starting from the date of surgery to the date of death or last follow-up. Survival

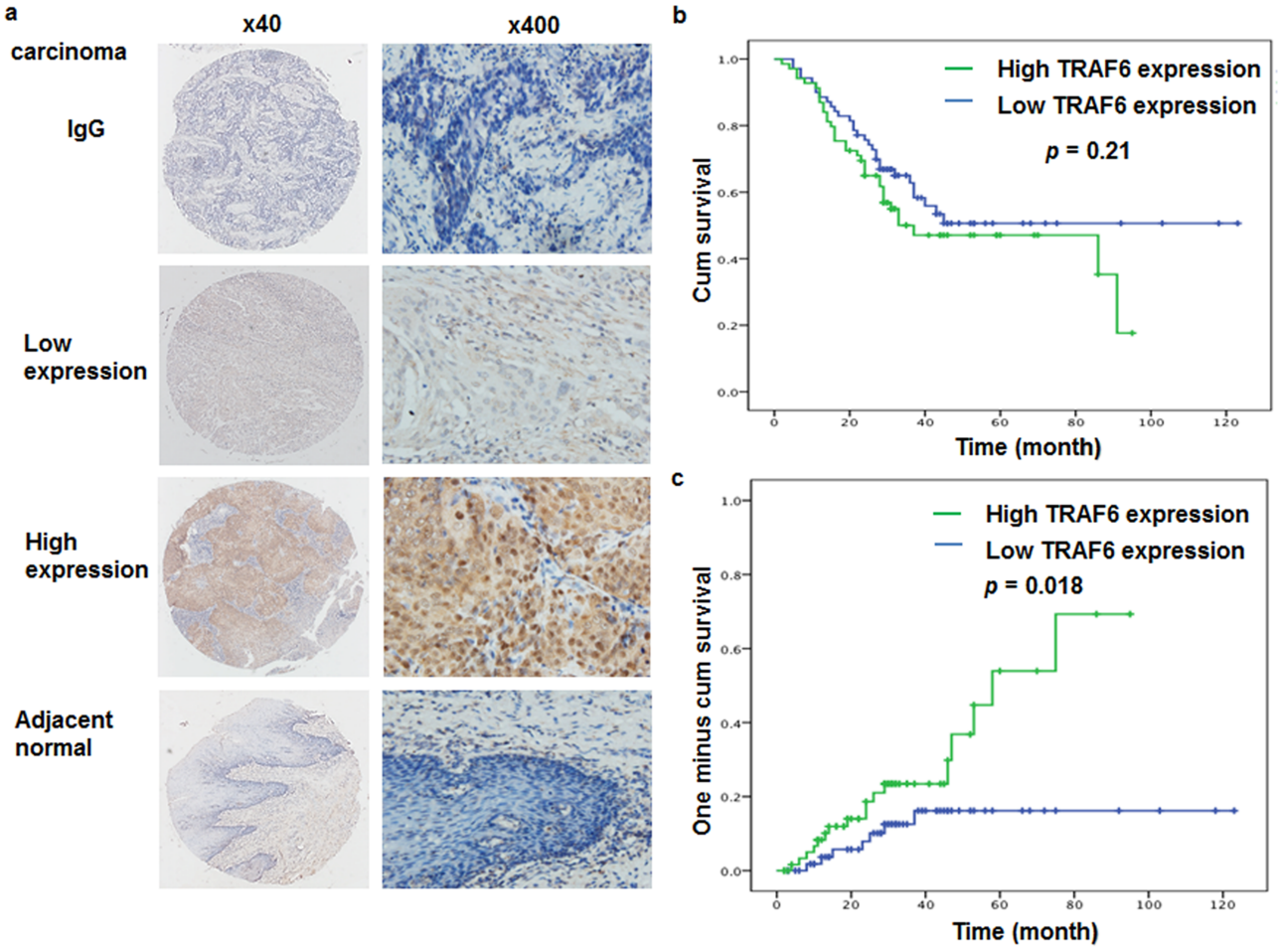

Figure 1. High expression of TRAF6 in esophageal carcinoma associated with recurrence.

curves for FoxO3a were plotted using Kaplan–Meier and compared using the log-rank test. Cox proportional hazard models were used for univariate and multivariate analyses to test clinical features for their associations with OS. In the multivariate Cox model, variables with $p<0.1$ from the univariate model were included. In addition to FoxO3a expression, the following variables were considered: age, gender, grading, and tumor location. Median time and hazard ratio (HR) were shown with 95% confidence interval (CI). All statistical analyses were performed using SPSS for Windows v.17.0 (SPSS, Chicago, IL, USA). All results were considered significant at two-sided $p<0.05$ value.

RESULTS

High Expression of TRAF6 in Esophageal Carcinoma Was Associated With Poor Prognosis

We analyzed the expression of TRAF6 in 146 patients with esophageal carcinoma using immunostaining analysis. Representative expression patterns in esophageal carcinoma samples are shown in Figure 1a. The expression of TRAF6 in cancerous tissues was much higher than that in adjacent normal tissues (Fig. 1a).

According to the expression levels of TRAF6 in esophageal carcinoma samples, all cases were distributed into two subgroups: low TRAF6 expression group ($n=70$) and high TRAF6 expression group ($n=76$) (Fig. 1a). Following the evaluation of immunohistochemical staining, the TRAF6 level was significantly associated with age and tumor differentiation (age: $p=0.013$, tumor differentiation: $p=0.036$). However, the TRAF6 level was not significantly associated with factors including gender, drinking history, smoking history, family cancer history, T stage, N stage, or TNM stage.

To further evaluate the association of TRAF6 expression with patients' prognosis, a log-rank test and Kaplan–Meier analysis were introduced to assess the effect of TRAF6 expression on the patient's survival and relapse. The univariate analysis revealed that TRAF6 was not significantly associated with OS, but significantly related to time to relapse ($n=146$, $p=0.018$) (Fig. 1b and c). Factors including drinking history, N stage, and TNM

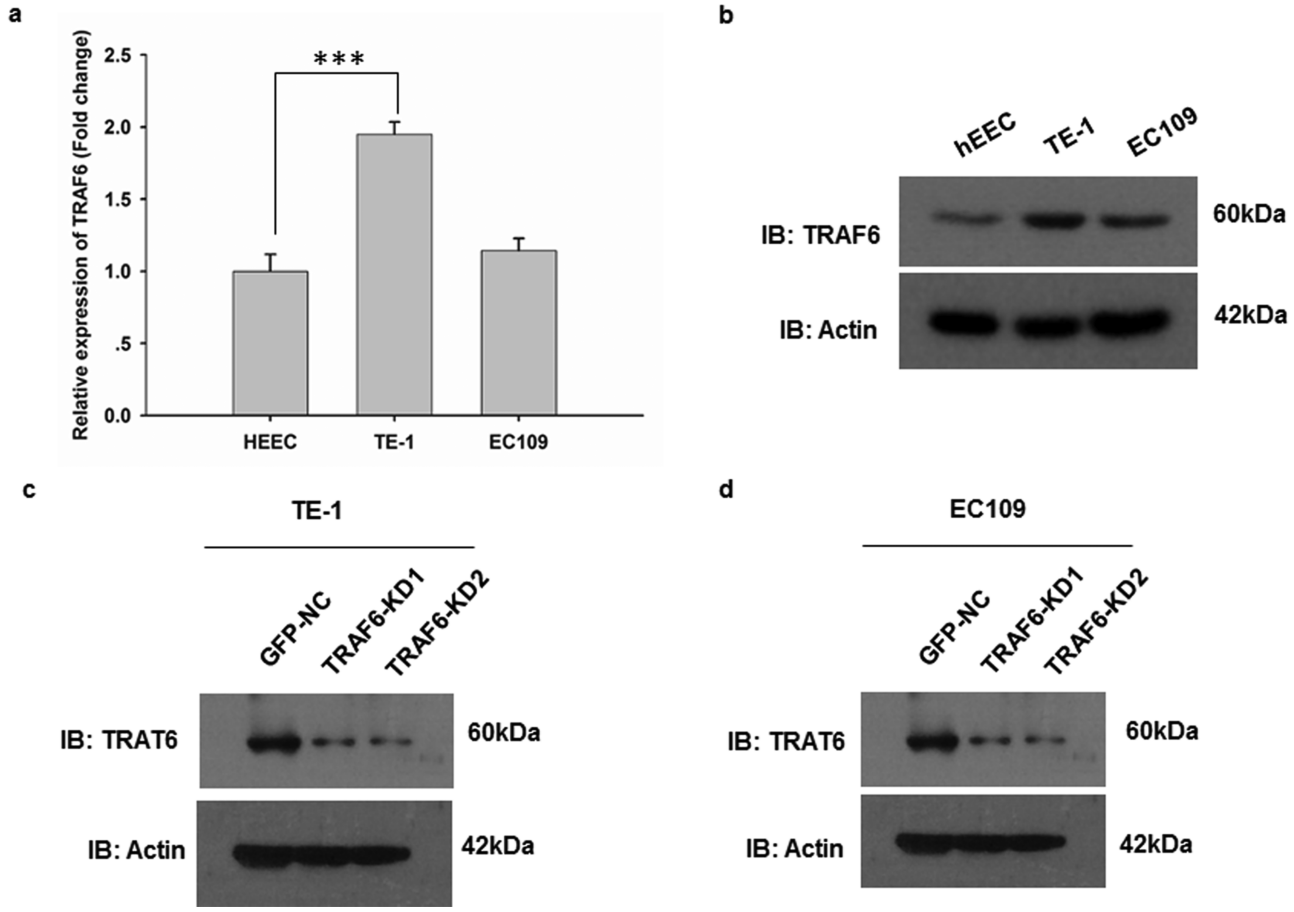

Figure 2. Knockdown of TRAF6 in esophageal carcinoma cells.

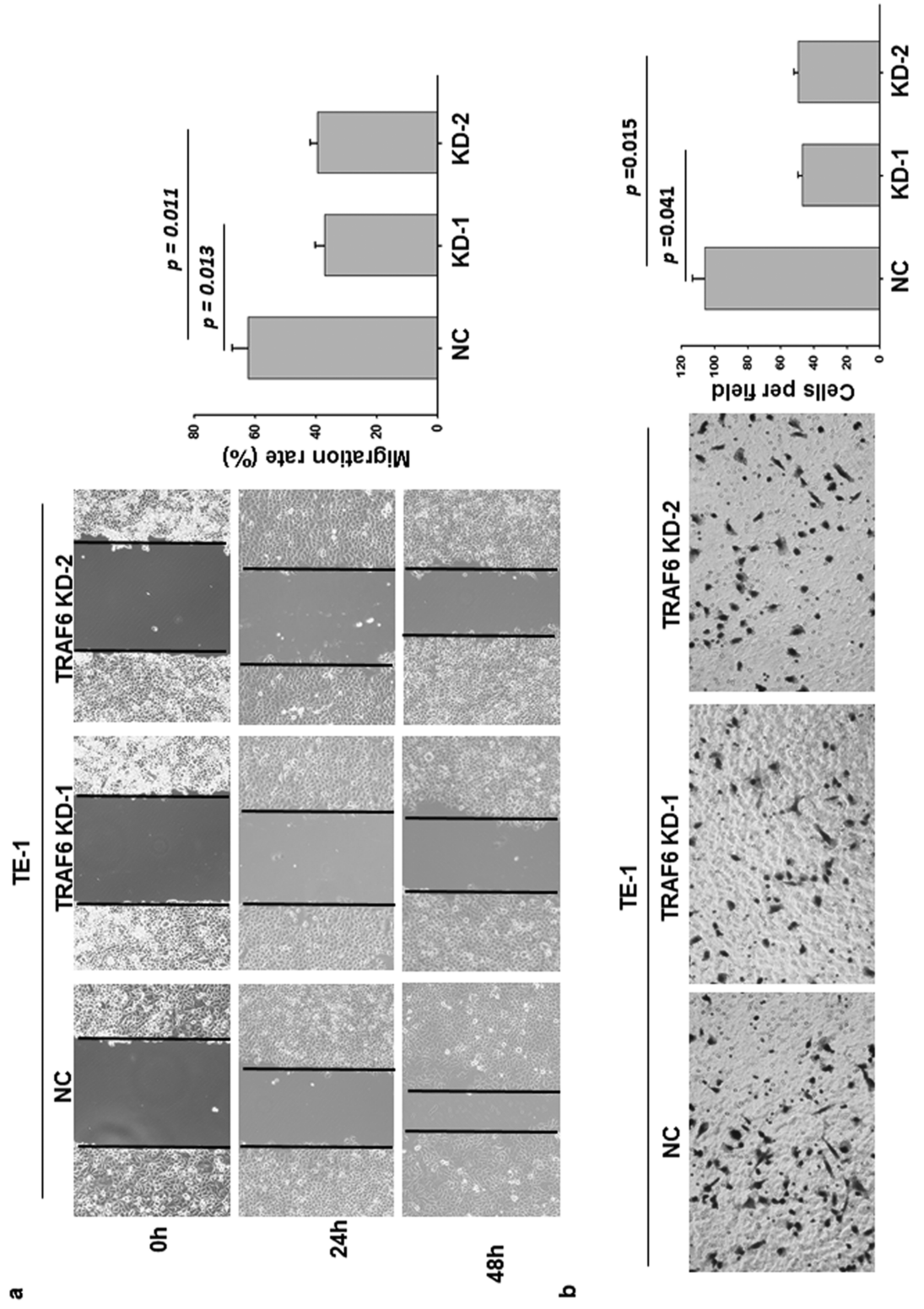

Figure 3. Knockdown of TRAF6 in esophageal carcinoma cell TE-1 inhibited cell migration and invasion abilities.

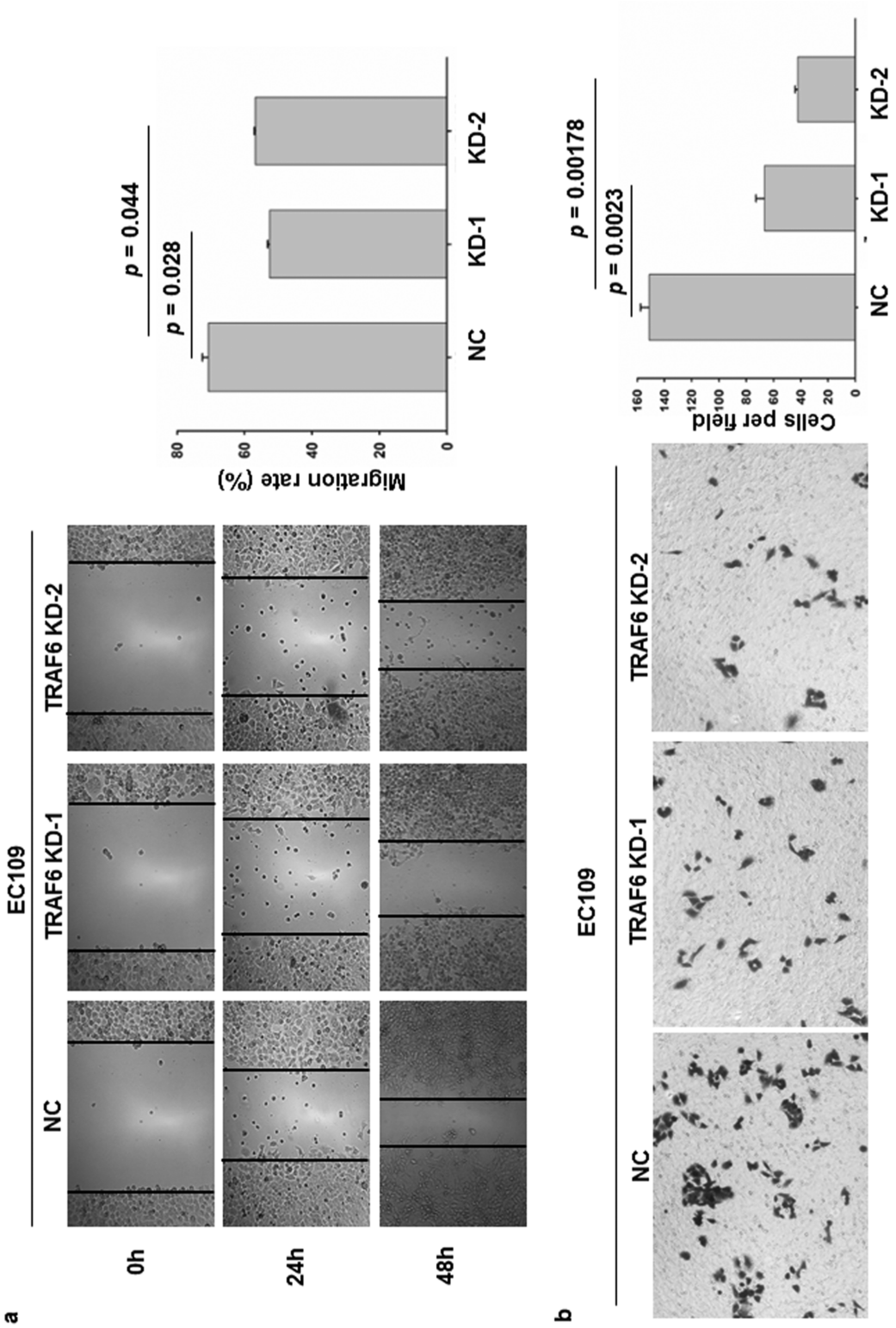

Figure 4. Suppression of TRAF6 in esophageal carcinoma EC109 inhibited cell migration and invasion abilities.

stage affected OS (drinking history: $p=0.033$, N stage: $p=0.000$, TNM stage: $p=0.000$).

Further, multivariate Cox regression analysis was also performed to explore whether TRAF6 was an independent prognostic factor for time to relapse. The analysis showed that TRAF6 expression was an independent prognosis factor (TRAF6: HR = 1.109, 95% CI = 1.003–1.226, $p=0.043$).

High Expression of TRAF6 in Esophageal Cancer Cells

We further analyzed TRAF6 expression in EC cells. The messenger RNA level of TRAF6 was analyzed by qRT-PCR. The protein expression level of TRAF6 was analyzed by Western blot. Compared to human normal esophageal epithelial cell (hEEC), TRAF6 was highly expressed in EC cells, TE-1, and EC109 (Fig. 2a and b). In order to further analyze the function of TRAF6 in EC cells, we constructed TRAF6 stably knocked-down EC cells through lentivirus-mediated target-specific shRNA. Two different shRNAs were designed to exclude off-target effects. The Western blot results confirmed the success in TRAF6 suppression in stably knocked-down TE-1 and EC109 cells (Fig. 2c and d).

TRAF6 Promotes the Migration and Invasion of Esophageal Cancer Cells

Metastasis is the major source of morbidity and mortality for patients with EC. To investigate the effects of TRAF6 on EC metastasis, both the migration and invasion abilities of EC cells were detected. Wound healing assay revealed that cells stably transfected with shRNA1 and shRNA2, but not scrambled shRNA, had significantly inhibited cell migration of EC cells (Figs. 3a and b and 4a and b). Moreover, the silencing of TRAF6 significantly decreased their invading capacity into Matrigel with the Transwell assay (Figs. 3c and d and 4c and d). Altogether, the tumor cell migration and invasion assay indicated that TRAF6 depletion significantly reduced the invasion and migration capabilities of EC cells.

TRAF6 Knockdown Inhibits EC Cell Migration and Invasion Through Targeting AEP and MMP2

To determine how TRAF6 influenced the invasive ability of EC cells, we detected some invasion-associated protein expression in TRAF6 knockdown cells and control cells. Interestingly, our Western blot analysis found that depletion of TRAF6 dramatically reduced AEP and MMP2 (Fig. 5). AEP and MMPs are known to facilitate cell invasion and metastasis by enzymatically degrading extracellular matrix components[23]. Thus, our results suggest that TRAF6 promotes migration and invasion through regulation of proteases (AEP and MMP2) in EC cells.

DISCUSSION

Despite recent advances in EC treatment, there has been no significant improvement in OS rate for advanced EC. New biomarkers are urgently needed to improve treatment of EC. Previous reports have indicated that TRAF6 was highly expressed in EC cancerous tissues[20–22]. Consistently, we find that TRAF6 was overexpressed in EC cancerous tissues and cells. Moreover, TRAF6 was independently associated with time to relapse. These observations suggest that TRAF6 may be a novel biomarker for EC recurrence. Hasan et al. found that

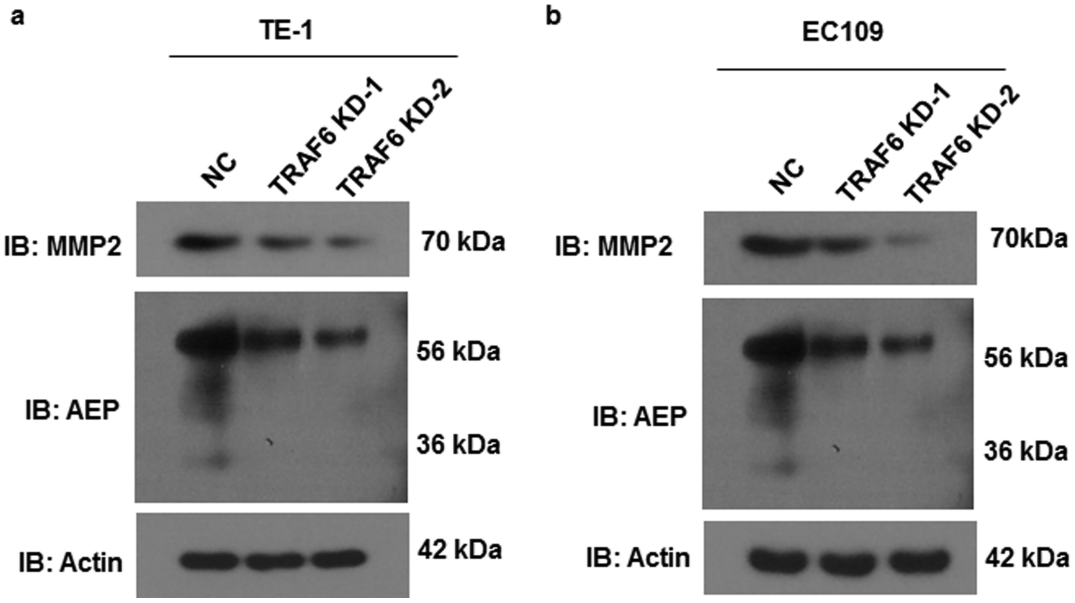

Figure 5. Inhibition of TRAF6 in esophageal carcinoma cells reduced MMP2 and AEP protein levels.

the biomarker signature score based on cytoplasmic β-catenin, nuclear c-Myc, nuclear DVL, and membrane α-catenin was associated with recurrence-free survival of EC patients[23]. Based on the literature, TRAF6 was first found to be related to recurrence in EC.

Previous studies have found that TRAF6 is an important gene involved in various processes of tumor progression. Zhao and colleagues reported that TRAF6 promoted the tumorigenesis and metastasis of esophageal squamous cell carcinoma[20,21]. Ma et al. showed that TRAF6 knockdown reduced EC109 cell proliferation and elevated apoptosis[22]. In our studies, we constructed two EC cells with stably knocked-down TRAF6 and found that TRAF6 silencing significantly reduced the migration and invasion abilities of both EC cells. Thus, our studies revealed new evidence for TRAF6 function in EC cells. However, the role of TRAF6 in EC progression should be further examined with an animal model in vivo.

The downstream substrates and associated pathways of TRAF6 have been found to include PI3K/AKT and the novel protease AEP. Nevertheless, it is important to identify more downstream factors of TRAF6 to further clarify its function in EC progression. Our data demonstrate that TRAF6 knockdown reduced the protein levels of MMP2 and AEP. Degradation of the extracellular matrix by cancer cells is an important process for direct invasion. AEP, currently the only known asparaginyl endopeptidase in the mammalian genome, is a member of the C13 family in the MEROPS database classification of peptidases, whereas all other lysosomal cysteine proteases identified to date are grouped in the C1 family[24]. The strict specificity of AEP to asparagine bonds is striking[25]. AEP has been found to play an important role in kidney physiology, immunity, atherogenesis, and bone metabolism[26–29]. Moreover, MMP2 is known to play an important role in ECM remodeling during the process of tumor invasion and metastasis[30]. Finding out new substrates of TRAF6 in EC is needed for further exploration.

In summary, we have uncovered a novel biomarker in EC recurrence, and TRAF6 might be of prognostic value and may be a therapeutic target. Targeting TRAF6 might have potential therapeutic value.

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