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TNFAIP3 overexpression is an independent factor for poor survival in esophageal squamous cell carcinoma

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Abstract. Tumor necrosis factor α induced protein 3 (TNFAIP3) is a protein that is induced by TNF-mediated NF-κB activation and has a dual function in regulating NF-κB. TNFAIP3 is associated with inflammatory carcinogenesis in many cancer types. However, the clinical significance of TNFAIP3 expression and function in esophageal squamous cell carcinoma (ESCC) has not yet been reported. We examined 149 ESCC tissue specimens to determine the clinical significance of TNFAIP3 by immunohistochemistry. Western blot analyses were used to detect TNFAIP3 expression in TE-1, TE-8, TE-15 and KYSE-70 ESCC cells and in Het-1A, a non-cancerous esophageal cell line. TNFAIP3 protein knockdown was conducted using small-interfering RNA to investigate its impact on cell proliferation, migration and invasion. Significant correlations between TNFAIP3 expression and differentiation (P=0.04) among clinicopathological characteristics of ESCC patients were demonstrated, and high TNFAIP3 expression was associated with poor survival (P=0.02). Moreover, multivariate analysis result showed that high TNFAIP3 expression was an independent factor for poor survival (P=0.04). *In vitro* analysis showed high expression of TNFAIP3 protein in TE-15 cells and low expression in Het-1A cells. Furthermore, the proliferation, migration and invasion of TE-15 cells after TNFAIP3 suppression by siRNA were significantly reduced. These findings suggest that TNFAIP3 protein may be an independent prognostic marker for poor survival, and a promising target for ESCC therapy.

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Abbreviations: ESCC, esophageal squamous cell carcinoma; TNFAIP3, tumor necrosis factor α induced protein 3; ZnF, zinc-finger; ABIN, A20 binding inhibitor of NF-κB activation; TNFAIP3-SNPs, TNFAIP3 in single nucleotide polymorphisms; IHC, immunohistochemical; UICC, International Union against Cancer; RT-PCR, reverse transcriptase PCR; siRNA, small-interfering RNA; OTU, ovarian tumor

Key words: TNFAIP3, A20, esophageal cancer, TE-15, siRNA

Introduction

Esophageal cancer is a serious malignancy and the eighth most common cancer worldwide, with an estimated 456,000 new cases, and the sixth most common cause of death from cancer with an estimated 400,000 deaths in 2012 (1). The global incidence of esophageal squamous cell carcinoma (ESCC) was 5.2 per 100,000 in the same year. Approximately 80% of cases of global ESCC occurred in the Central and East Asian region (2). The overall 5-year survival rate for ESCC ranges from 15 to 25% (3). Biomarker discovery for the malignancy could potentially lead to earlier diagnosis as well as allowing the monitoring of cancer recurrence (4).

Tumor necrosis factor α induced protein 3 (TNFAIP3 or A20) was first identified as a gene that is activated in response to TNFα in human umbilical vein endothelial cells (5). TNFAIP3 protein is composed of seven Cys2/Cys2 zinc-fingers (ZnFs) (6) that are induced by TNF-mediated NF-κB activation (7), and has a dual-function as a ubiquitin-editing enzyme to regulate NF-κB through several molecules involved in the NF-κB pathway (8,9).

TNFAIP3 was originally characterized as a protein that protects cells from the cytotoxic effect of TNF (10), and regulates TNF receptor signals by interactions with TNF receptor-associated factor-2 (11), interleukin 1, A20 binding inhibitor of NF-κB activation (ABIN) (12), inhibitor of NF-κB kinase γ (13), and stress-activated protein kinase (14). TNFAIP3 was also demonstrated to interact with TXBP151 protein to mediate the anti-apoptotic process through cleavage of caspase-3, -6 and -7 (15), suggesting that overexpression of TNFAIP3 is correlated with inflammatory and malignant diseases (16).

Recently, we reported the correlation of TNFAIP8 overexpression and cancer progression and poor prognosis in ESCC clinical samples. TNFAIP8 was also demonstrated as an

effective therapeutic target for ESCC. TNFAIP8 is an apoptosis regulator and contains a death-effector domain that is also induced by NF-κB activation (17). Although TNFAIP3 and -8 have a different mechanism, both were reported to play a role in multiple myeloma (18). As with TNFAIP8, TNFAIP3 may also be a promising therapeutic target for malignant diseases.

Overexpression of TNFAIP3 was also found in breast tumor (19), pancreatic cancer (20), hepatocellular carcinoma (21) and bladder cancer (22). Also, single nucleotide polymorphisms in TNFAIP3 (TNFAIP3-SNPs) were reported to be associated with advanced disease stage and survival in surgically-treated esophageal adenocarcinoma and squamous cell carcinoma (23). However, the clinical study and function of TNFAIP3 in malignant disease is very limited, especially in ESCC. In this study, we evaluated the correlation between TNFAIP3 expression and cancer progression in ESCC clinical samples. We also investigated the TNFAIP3 function in ESCC cells *in vitro*.

Materials and methods

Patients and specimens. Surgical specimens for immunohistochemical (IHC) study were obtained from 149 ESCC patients (134 males and 15 females) who underwent potentially curative surgery; no evidence of residual tumors and the resected margins were free of tumors by microscopic examination (R0) at the Department of General Surgical Science, Gunma University, between 2000 and 2010, after obtaining written informed consent. The patients were aged from 41 to 83 years (mean, 64.1 years). Tumor stage and disease grade of clinical samples were classified according to the 6th edition of the TNM classification of the International Union against Cancer (UICC). The tumor differentiation evaluation was based on the histological criteria outlined by the Japanese Society for Esophageal Disease.

Surgical specimens for RNA samples were obtained from 83 ESCC patients (72 males and 11 females) that were a subset of IHC samples. The patients were aged from 42 to 83 years (mean, 65.2 years). Normal tissues were obtained far from the margin of the cancer in surgical specimens. All specimens for RNA extraction were immediately frozen in liquid nitrogen and stored at -80˚C until RNA extraction.

None of the patients had received radiotherapy or chemotherapy prior to surgery, nor did any have hematogenous metastases at the time of surgery. Patients who had undergone non-curative surgery and/or who had received inadequate follow-up were excluded from the study.

Immunohistochemistry. Resected specimens were fixed with 10% formaldehyde, embedded in paraffin blocks, cut into 4 μm thick sections, and mounted onto platinum pro-micro slide glass (Matsunami Glass Ind., Ltd., Osaka, Japan). We examined sections containing portions of both tumor and its normal esophageal epithelium as inner control. Each section was deparaffinized, rehydrated, and incubated with fresh 0.3% hydrogen peroxide in methanol for 30 min at room temperature to block endogenous peroxidase activity. After being rehydrated through a graded series of ethanol concentrations, the sections were autoclaved in 10 mM citrate buffer (pH 6.0) at 120˚C for 2 min and then cooled to 30˚C. After rinsing the sections in 0.1 M phosphate-buffered saline (PBS; pH 7.4), non-specific binding sites were blocked by incubation with 10% normal goat serum for 30 min. The sections were then incubated at 4˚C overnight with rabbit anti-TNFAIP3 polyclonal antibody (1:150; Abcam, Cambridge, UK) in PBS containing 1% bovine serum albumin. Negative controls were obtained by absence of the specific primary antibody. The sections were then washed in PBS and incubated with biotinylated anti-rabbit IgG for 30 min at room temperature. IHC was performed using a Histofine streptavidin-biotin peroxidase (SAB-PO) complex solution kit (Nichirei Co., Tokyo, Japan). The sections were then lightly counterstained with Mayer's hematoxylin and mounted.

The intensity and area of TNFAIP3 staining in tumor tissues was scored between 0-3, as follows; 0, no staining, 0%; 1, weak, <30%; 2, moderate, 30-60%; and 3, strong intensity, >60%. Low TNFAIP3 expression was scored 0-1, while high TNFAIP3 expression was 2-3. TNFAIP3 staining evaluation was performed by three experienced researchers well trained in pathology, in a blinded manner; i.e., they did not have any knowledge of the clinical or pathological backgrounds of the patients, or the assessment of each other.

RNA extraction and quantitative real-time reverse transcriptase PCR. Total RNA was extracted from the tissue and cells using the RNeasy plus Mini kit (Qiagen, Hilden, Germany). The quantity of isolated RNA was measured using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). cDNA for *TNFAIP3* mRNA quantitative real-time reverse transcriptase PCR (RT-PCR) was synthesized from 1 μg total RNA with the Omniscript Reverse Transcriptase kit (Qiagen) in a reaction volume of 20 μl (60 min at 37˚C and 5 min at 93˚C before being put on ice). The *TNFAIP3*-specific oligonucleotide primers were designed as follows: *TNFAIP3* forward, 5'-TGCACACTGTGTTTCATCGAC-3'; reverse, 5'-ACGCTGTGGGACTGACTTTC-3'. *GAPDH* (258 bp) forward, 5'-AAGGTGAAGGTCGGAGTCAAC-3'; reverse, 5'-CTTGATTTTGGAGGGATCTCG-3'. PCR amplification to quantify the levels of *TNFAIP3* and *GAPDH* mRNA in the clinical samples was performed using a Light cycler 480 Real-Time PCR system and the LightCycler 480 SYBR Green I Master kit (Roche Applied Science, Mannheim, Germany). The amplification conditions consisted of initial denaturation at 95˚C for 10 min followed by 40 cycles of denaturation at 95˚C for 10 sec, annealing at 60˚C for 10 sec, and elongation at 65˚C for 10 sec. All of the samples, expression of TNFAIP3 mRNA was calculated by dividing the quantity of TNFAIP3 mRNA with the quantity of GAPDH mRNA. To investigate the correlation of TNFAIP3 mRNA expression levels with clinicopathological features, we divided the tumor TNFAIP3 mRNA expression value with the corresponding non-cancerous ones in each samples. The results of samples with TNFAIP3 mRNA expression value higher than the median value of all samples was considered high expression, while that lower than the median value was low expression.

Cell lines. Four esophageal cancer cell lines were established from squamous cell carcinoma KYSE-70, TE-1, TE-8, TE-15 cell lines, and the non-cancerous immortalized esophageal cell line Het-1A, was used. Het-1A, TE-1, TE-8, TE-15 and

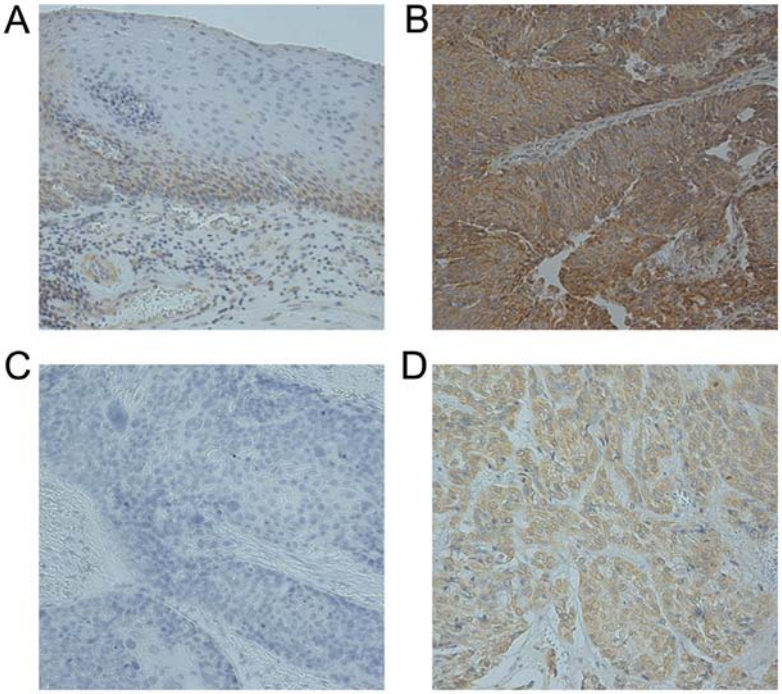

Figure 1. Immunohistochemical staining of TNFAIP3 in ESCC and normal esophageal epithelium tissues. (A) TNFAIP3 expression in a representative epithelial tissue section of esophagus. (B) High TNFAIP3 expression in a representative ESCC tissue section. (C) TNFAIP3 negative control in a representative ESCC tissue section was prepared using non-immune rabbit IgG at the same dilution as the primary antibody. (D) Low TNFAIP3 expression in a representative ESCC tissue section. All images were captured using a phase-contrast microscope (x200).

KYSE-70 cells were provided from the American Type Culture Collection (Manassas, VA, USA), JCRB Cell Bank, and RIKEN BioResource Center (Ibaraki, Japan) through the National Bio-Resource Project of the MEXT, Japan. TE-1, and TE-15 cells are well-differentiated ESCC primary lesion cells, TE-8 cells are moderately differentiated ESCC primary lesion cells, while KYSE-70 cells are from a poorly-differentiated ESCC sample. All cell lines were cultured in RPMI-1640 (Wako, Osaka, Japan) containing 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). The cells were cultured in a humidified 5% CO_2 incubator at 37˚C.

Western blot analyses. Het-1A, KYSE-70, TE-1, TE-8 and TE-15 cells ($1x10^6$ cell/ml) were washed twice in ice-cold medium and cell lysates were prepared after solubilizing cells with PRO-PREP Protein Extraction Solution (iNtRON Biotechnology, Gyeonggi-do, Korea). Protein concentrations of the lysates were determined with a Pierce BCA protein assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) using bovine serum albumin as a standard. Forty microgram of each extract was subjected to electrophoresis on a NuPAGE Novex 4-20% Bis-Tris gel (Thermo Fisher Scientific) and the proteins were electrotransferred to a Hybond ECL (7x8 cm) membrane (GE Life Science Healthcare, Little Chalfont, UK). The membranes were then incubated overnight at 4˚C with rabbit polyclonal antibody against TNFAIP3 (1:1,000; Abcam) and mouse monoclonal antibody against β-actin (1:2,000; Sigma-Aldrich, St. Louis, MO, USA). Bands on the membrane were detected using an Image Quant LAS4000 (GE Life Science) with the aid of an enhanced chemiluminescence detection system.

Small interfering RNA. *TNFAIP3* small interfering RNA (siRNA) (hTNFAIP3_#1: sense 5'-CAAAGUUGGAUGAAGC UAAtt and antisense 5'-UUAGCUUCAUCCAACUUUGtt, hTNFAIP3_#2: sense 5'-GCACCAUGUUUGAAGGAUAtt and antisense 5'-UAUCCUUCAAACAUGGUGCtt and hTNFAIP3_#3: sense 5'-GAGCAGGAGAGGAAAGAUAtt and antisense 5'-UAUCUUUCCUCUCCUGCUCtt) siRNAs were purchased from GeneDesign (Osaka, Japan). TE-15 cells were seeded in 6-well flat-bottomed microtiter plates at a density of $1.5x10^5$ cells per well in a volume of 2 ml and incubated in a humidified atmosphere (37˚C and 5% CO_2). After incubation, TE-15 cells were treated with siRNAs according to the manufacturer's instructions to final concentration 30 nM per well, by adding Opti-MEM I Reduced-Serum Medium liquid (Thermo Fisher Scientific) mixed with Lipofectamine RNAi MAX (Thermo Fisher Scientific). The experiments were then performed after 48 h incubation.

Cell proliferation assay. Cell proliferation analysis was performed using TE-15 cells transfected with *TNFAIP3* siRNA; 100 μl of a cell suspension ($1x10^4$ cells) was seeded into each well of a 96-well plate (Falcon, Franklin Lakes, NJ, USA) and incubated at 37˚C overnight. Cell viability was determined using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Proliferation index were assessed as absorbance at 450 nm (OD_{450}) the reference wavelength at 620 nm was read and the results were derived from three sets of triplicate experiments.

Wound healing assay. To determine the effect of TNFAIP3 on esophageal cell migration, a wound-healing assay was

performed in TE-15 cells transfected with *TNFAIP3* siRNAs. The cells were seeded in 6-well plates and incubated until 80% confluence. A wound was made through the monolayer using a sterile 200 μl pipette tip and cell debris removed by washing the cells with PBS three times. Wounds were observed under a microscope and measured over a time course to calculate the migration rate according to the following formula: percentage wound healing for 24 and 48 h = [(wound length at 0 h) - (wound length at 24 or 48 h)]/(wound length at 0 h) x 100. The experiments were performed three times with triplicate samples.

Cell invasion assay. Cell invasion was examined using the BD BioCoat Matrigel invasion chamber (8.0 μm, BD Bioscience, San Jose, CA, USA) according to the manufacturer's instructions. Five hundred microliter containing 2.5×10^4 TE-15 cells transfected with *TNFAIP3* siRNA were added to each invasion chamber. After incubation for 12 h, the cells were stained with a Diff-Quick kit (Sysmex Corp., Kobe, Japan), and then observed and counted under the microscope. The parental groups were used for normalization. All samples were tested twice in triplicate.

Statistical analysis. The χ^2 test and t-test were used to assess the statistical significance of the correlations between TNFAIP3 expression and clinicopathological parameters. Kaplan-Meier curves were generated for disease-specific survival. In addition, univariate and multivariate survival analyses were performed using Cox's proportional hazards regression model. Statistical analyses were performed using JMP5.0 software (SAS Institute Inc., NC, USA). Differences were considered statistically significant when the P-value was <0.05.

Results

TNFAIP3 protein and mRNA expression levels in ESCC tissue samples. In normal esophageal epithelium, expression of TNFAIP3 was low in the basal layer of the epithelium (Fig. 1A). TNFAIP3 expression was localized in the cytoplasmic components of tumor cells (Fig. 1B and D). The expression of TNFAIP3 was investigated by IHC in 149 ESCC specimens, and we found that TNFAIP3 protein expression was high in 71 specimens (47.65%).

TNFAIP3 high expression is correlated with differentiation of ESCC pathological features in protein level, but not in mRNA level. The correlations between TNFAIP3 expression and the clinicopathological characteristics of ESCC patients (age, gender, differentiation, TNM stage, tumor depth, lymph node metastasis, distant metastasis, lymphatic invasion, and venous invasion) that were investigated by IHC are shown in Table I. A significant correlation between TNFAIP3 expression and tumor differentiation status (P=0.041) was identified, whereas there were no significant correlation between TNFAIP3 expression and age (P=0.137), or gender (P=0.935), TNM stage (P=0.603), tumor depth (P=0.381), lymph node metastasis (P=0.534), distant metastasis (P=0.299), lymphatic invasion (P=0.391), or venous invasion (P=0.075). However, no significant correlations were found between *TNFAIP3* mRNA expression and the clinicopathological characteristics of ESCC patients or patient survival rates (data not shown).

Table I. Correlations between TNFAIP3 expression and clinicopathological features.

Characteristics	TNFAIP3 low n=78	TNFAIP3 high n=71	P-value
Age (years) (mean ± SD)	63.14±0.90	65.09±0.94	0.137
Gender			
Male	70	64	0.935
Female	8	7	
Differentiation			
Well	8	17	0.041[a]
Moderate	41	37	
Poor	29	17	
TNM stage			
I	18	60	0.603
II, III, IV	60	52	
Tumor depth			
T1	36	24	0.381
T2	9	8	
T3	31	35	
T4	2	4	
Lymph node metastasis			
N0	28	29	0.534
N1	50	42	
Distant metastasis (LYM)			
M0	62	61	0.299
M1	16	10	
Lymphatic invasion			
Negative	14	10	0.391
Positive	64	61	
Venous invasion			
Negative	23	13	
Positive	55	58	0.075

[a]P<0.05.

TNFAIP3 high expression is correlated with poor survival as independent factor. The disease-specific survival rate of the ESCC patients with high TNFAIP3 expression was significantly poorer than that of the patients with low TNFAIP3 expression (P=0.025; Fig. 2). In univariate analysis, high TNFAIP3 expression was found to be a significant prognostic factor for poor survival, in addition to tumor depth, the presence of lymph node metastasis, distant metastasis (cervical lymph node metastasis, not distant organ metastasis), lymphatic invasion, and venous invasion. Moreover, multivariate analysis of the six factors revealed that the presence of distant metastasis was significant (P=0.008) and showed that positive TNFAIP3 expression was an independent prognostic factor (P=0.021; Table II).

Table II. Univariate and multivariate analyses of survival in 149 ESCC patients.

	Univariate analysis		Multivariate analysis	
Characteristic	HR (95% CI)	P-value	HR (95% CI)	P-value
TNFAIP3 expression				
Low vs high	1.34 (1.03-1.76)	0.025[a]	1.37 (1.04-1.82)	0.021[a]
Tumor depth				
T1 vs T2-4	0.65 (0.48-0.87)	0.003[a]	0.97 (0.69-1.33)	0.879
Lymph node metastasis				
N0 vs N1	0.52 (0.35-0.73)	0.0001[a]	0.68 (0.43-1.00)	0.056
Distant metastasis (LYM)				
M0 vs M1	0.56 (0.42-0.74)	0.0001[a]	0.66 (0.49-0.89)	0.008[a]
Lymphatic invasion				
Negative vs positive	2.34 (1.30-5.79)	0.001[a]	1.05 (0.45-2.99)	0.908
Venous invasion				
Negative vs positive	2.42 (1.46-4.90)	0.0001[a]	1.63 (0.86-3.76)	0.139

HR, hazard ratio; CI, confidence interval. [a]P<0.05.

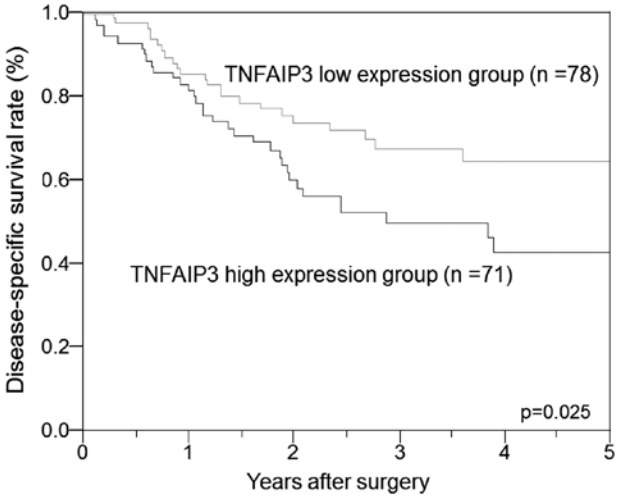

Figure 2. Disease-specific 5-year survival rate according to TNFAIP3 expression. The disease-specific 5-year survival rates of the ESCC patients in the high TNFAIP3 expression group was significantly lower than that in the patients with low TNFAIP3 expression.

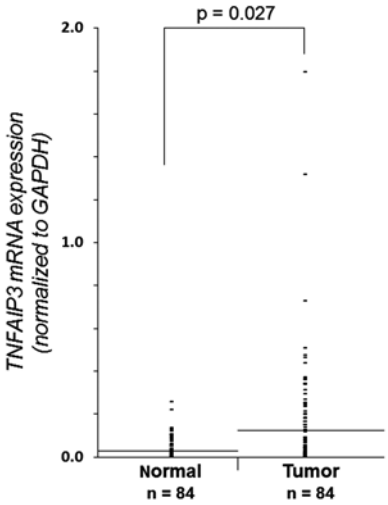

Figure 3. *TNFAIP3* mRNA expression in ESCC patient samples. Quantitative real-time RT-PCR analysis of *TNFAIP3* mRNA expression in ESCC samples and adjacent normal tissues. *TNFAIP3* expression in tumor tissues was significantly higher than that in the normal tissues.

TNFAIP3 mRNA expression in ESCC tissues was significantly higher than in non-cancerous tissue. Whereas, *TNFAIP3* mRNA expression was determined in clinical samples from 83 patients using real-time RT-PCR, and 53 patients (63.85%) showed higher *TNFAIP3* mRNA expression levels in their ESCC tissue specimens (Fig. 3). The mean *TNFAIP3* mRNA level in the ESCC tissue was significantly higher than that in the corresponding non-cancerous tissue (P=0.027).

Expression of TNFAIP3 protein in ESCC cells and non-cancerous immortalized esophageal cells and knockdown of TNFAIP3 in TE-15 cells. We examined TNFAIP3 protein expression in four ESCC cell lines (KYSE70, TE-1, TE-8 and TE-15) and the non-cancerous immortalized esophageal cell line Het-1A. TNFAIP3 protein was expressed at low levels in Het-1A and TE-8 cells, whereas it was moderately expressed in KYSE70 and TE-1 cells and showed extremely high expression in TE-15 cells (Fig. 4A). Based on these results, we used TE-15 cells to analyze cell behavior following knockdown of *TNFAIP3*.

To determine the contribution of high expression levels of TNFAIP3 in ESCC cells, we used siRNA to knock down *TNFAIP3* expression in TE-15 cancer cells. The suppression of *TNFAIP3* by siRNAs was checked using western blotting (Fig. 4B). Since *TNFAIP3* siRNA3 did not show any significant reduction, we used *TNFAIP3* siRNA1 and siRNA2 for the subsequent analysis.

Depletion of TNFAIP3 expression inhibits cell proliferation, migration, and invasion. TE-15 cells treated with *TNFAIP3*

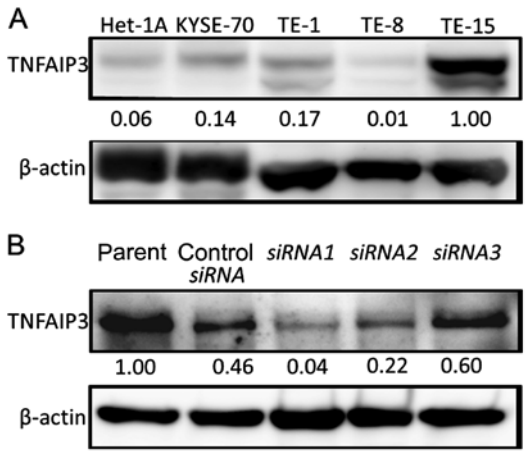

Figure 4. TNFAIP3 protein expression and knockdown in ESCC cell lines. (A) TNFAIP3 protein expression in Het-1A, KYSE-70, TE-1, TE-8, and TE-15 cells. Low TNFAIP3 protein levels in Het-1A cells and high TNFAIP3 levels in TE-15 cells were detected by western blot analysis. TNFAIP3 expression was normalized to β-actin expression. (B) TNFAIP3 protein levels in TE-15 cells treated with siRNA. TNFAIP3 expression was suppressed by *TNFAIP3* siRNA1 and *TNFAIP3* siRNA2 treatment. TNFAIP3 expression was normalized to β-actin expression.

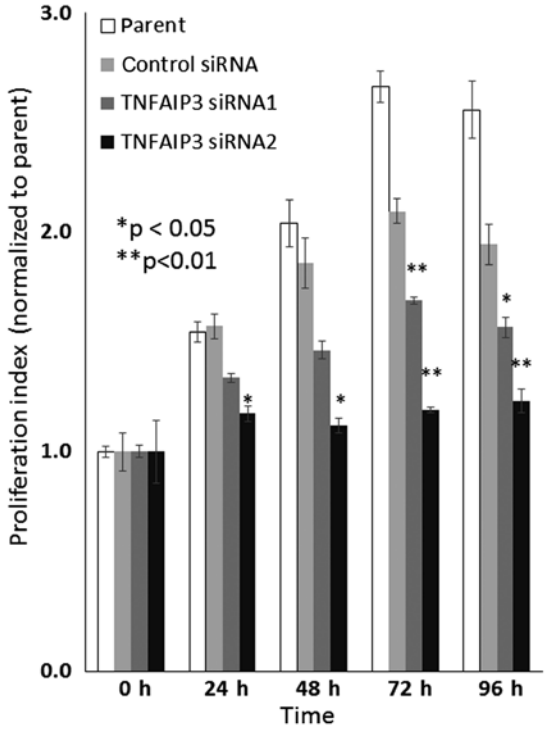

Figure 5. Effect of *TNFAIP3* siRNA knockdown in TE-15 cells. Proliferation of TE-15 cells treated with *TNFAIP3* siRNA1 was observed after 72 and 96 h, and those treated with *TNFAIP3* siRNA2 were observed after 24, 48, 72, and 96 h (*P<0.05, **P<0.01). The proliferation index was normalized to untreated TE-15 cells.

siRNA1 and siRNA2 showed a significant reduction in their cell proliferation rate compared with cells transfected with the negative control siRNA and the parental group (Fig. 5).

A significant reduction in cell migration ability was demonstrated in TE-15 cells that were treated with *TNFAIP3* siRNA1 and siRNA2 compared with cells transfected with the negative control siRNA and the parental group (Fig. 6).

The weakening of the cell ability to invade was shown in TE-15 cells treated with *TNFAIP3* siRNA1 and siRNA2 compared with cells transfected with the negative control siRNA and the parental group (Fig. 7). Based on the *in vitro* experiment results above, the depletion of TNFAIP3 protein expression seems to decrease cancer cell proliferation, migration and invasion ability.

Discussion

In the present study, we found that high expression of TNFAIP3 in ESCC clinical samples was significantly correlated with tumor differentiation that is associated with tumor growth and spread. The disease-specific survival rates of patient with high TNFAIP3 expression were significantly poorer than those of patients with low TNFAIP3. Moreover, multivariate analysis showed that positive TNFAIP3 expression was an independent prognostic factor for poor survival. According to clinical results, we investigated whether the TNFAIP3 overexpression accelerate tumor growth and spread enhancing the ability of tumor cells to migrate and invade. *In vitro* analysis showed that proliferation, migration and invasion were significantly reduced following *TNFAIP3* siRNA knockdown compared with the control groups. Our results indicated that TNFAIP3 plays a role in enhancing the ability of cells to expand, that might be the reason why of the tumor appears to grow slowly in the primary tumor, but actually tumor cells migrate to other organs.

TNFAIP3 is an anti-inflammatory signaling molecule that was reported to protect cells from cytotoxic effects (10). Since TNFAIP3 was found to regulate NF-κB signaling through ubiquitination to form germline polymorphisms and somatic mutations, TNFAIP3 has been linked to multiple diseases. TNFAIP3-SNPs that might reduce TNFAIP3 function and expression were linked with multiple inflammatory diseases such as systemic lupus erythematosus, rheumatoid arthritis, psoriasis, and type 1 diabetes (16). TNFAIP3 with missense mutations was reported in breast cancer cells at 14-3-3 binding sites by changing the crucial serine residue to arginine (24). TNFAIP3 mutations cause overexpression and confer an anti-apoptotic function on TNFAIP3 to mediate RIP1 ubiquitination and hence inhibit caspase-8 cleavage in hepatocellular carcinoma cells (21). In endothelial cells, TNFAIP3 overexpression was showed to restrict TNF- and FAS-induced apoptosis by preventing subsequent activation of the apical caspases-8 and -2 to the downstream effectors (25). High TNFAIP3 expression caused by TNFAIP3 mutations were also reported in glioma stem cells (26). However, no study has been reported on high expression of TNFAIP3 associated with mutations in ESCC.

A previous study reported that germline TNFAIP3-SNPs in esophageal cancer had a relationship with AJCC disease stages, lymph node metastasis, and recurrence (23); however, in the present study, the overexpression of TNFAIP3 protein was correlated with differentiation. TNFAIP3 expression was high in well-differentiated ESCC and low in poorly and moderately differentiated cancer in the present study. This finding was reinforced by the findings *in vitro* that showed high TNFAIP3 expression was found in TE-15 cells, which are well-differentiated ESCC cells. Although these studies

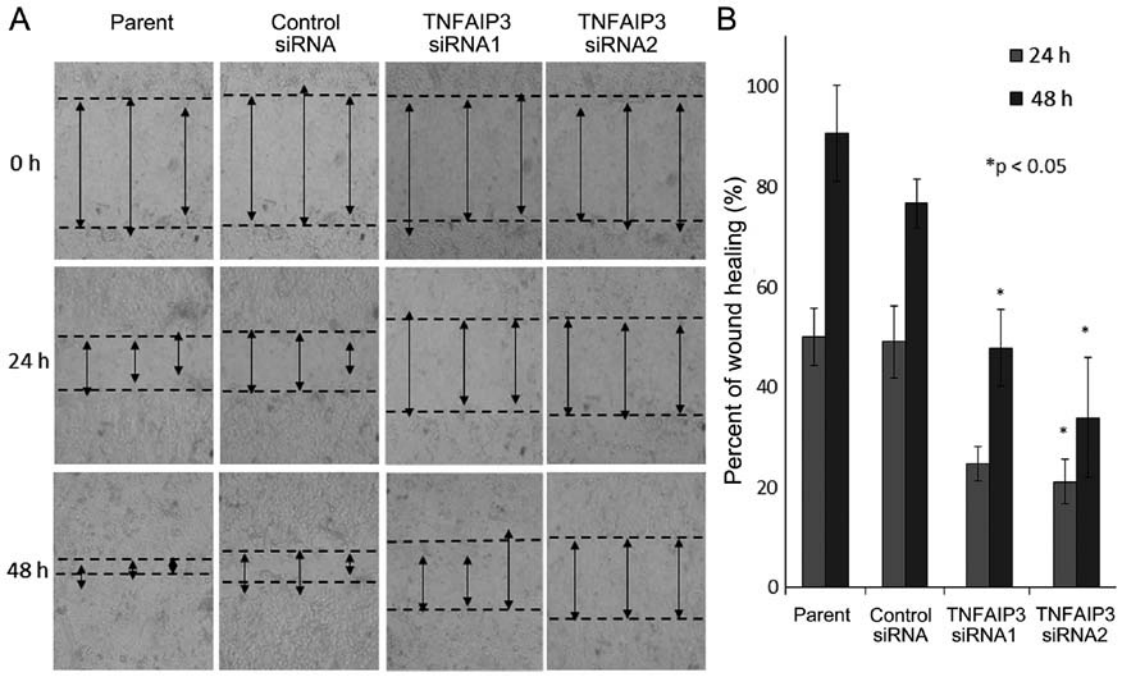

Figure 6. Wound healing assay was performed in TE-15 cells after *TNFAIP3* siRNA treatment. (A) Wound healing assay of untreated TE-15 cells (parental) and TE-15 cells treated with control siRNA, *TNFAIP3* siRNA1 and *TNFAIP3* siRNA2 at 0, 24 and 48 h. (B) Wound healing of TE-15 cells was significantly reduced in cells treated with *TNFAIP3* siRNA1 treatment after 48 h and *TNFAIP3* siRNA2 treatment after 24 and 48 h (P<0.05).

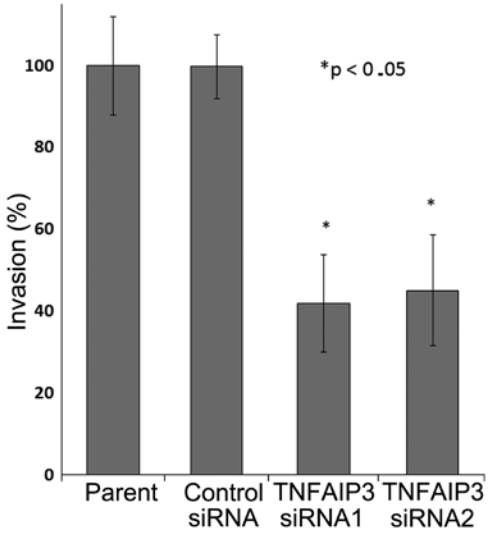

Figure 7. Invasion assays were performed in TE-15 cells after *TNFAIP3* siRNA treatment. Significant reduction in the invasive ability of TE-15 cells treated with *TNFAIP3* siRNA1 and *TNFAIP3* siRNA2 was observed (P<0.05). Invasion in the treated cells (%) was normalized to untreated TE-15 cells.

are different, the prediction obtained from previous studies demonstrated that TNFAIP3 regulates NF-κB signaling (8,16), with downstream effects on TGF-β signaling (27) and Wnt/β-catenin signaling (28), to influence tumor differentiation. However, for details of the mechanism further investigation is required.

In head and neck squamous cell carcinoma, TNFAIP3 was expressed in poorly differentiated and undifferentiated, but not in well-differentiated cancers in mRNA level (29). However, there was no significance difference between *TNFAIP3* mRNA levels and clinicopathological factors in this study, including tumor differentiation. Nonetheless, TNFAIP3 protein expression has a relationship with clinicopathological factors such as tumor differentiation. A previous study reported that a truncation mutant form of TNFAIP3 containing four ZnFs is responsible for the ubiquitination that can regulate TNFAIP3 protein expression levels (6). TNFAIP3 consists of two ubiquitin-editing domains, an N-terminal deubiquitinating domain of the ovarian tumor (OTU) family and a C-terminal ubiquitin ligase domain. In a previous study, the N terminal domain was shown to deubiquitinate K63-polyubiquitinated RIP1. The C-terminal ZnF-containing domain functions as a ubiquitin ligase that catalyzes K48-linked RIP1, targeting RIP1 for proteasomal degradation. Wild-type TNFAIP3 disassembles K63-linked ubiquitin chains on RIP1 and increases K48-linked RIP1 ubiquitination. Furthermore, TNFAIP3 with a ZnF4 mutation at the OTU-domain inhibits the disassembling of K63-linked ubiquitin chains on RIP1 and diminishes the ability of TNFAIP3 to ubiquitinate RIP1 with K48-linked ubiquitin chains (8,9). The dual-ubiquitination of mutant TNFAIP3 is predicted to cause differences in TNFAIP3 mRNA and protein expression levels in our study (16).

TNFAIP3-SNP was also found to be an independent prognostic factor, but AC and CC genotypes had a relationship with disease-free and overall survival in esophageal cancer (23). Even though it is not on the same condition that deserves to be compared, the present study revealed that TNFAIP3 was found to be an independent prognostic factor, and ESCC patients with high expression of TNFAIP3 had significantly poorer disease-specific survival than those patients with low expression ones. Although TNFAIP3 was not significantly correlated with metastases (lymph node and distant) or invasion (lymphatic and venous) in ESCC clinicopathological

features, but in multivariate analysis TNFAIP3 showed significance (P=0.021) after distant metastases (P=0.008). Since activation of JAK/STAT pathway is predicted to play roles in malignancies, including tumor proliferation, invasion and metastases, activation of STAT3 was also considered a responsible mechanism for TNFAIP3 becoming an independent prognostic factor in the present study (30). According to a previous study, SNPs of TNFAIP3 in the C-allele and alteration of the binding of transcription factors reduce TNFAIP3 expression (16). It means this study results would be reasonable if that happens is the opposite of the previous results studied in TNFAIP3-SNP.

From our clinicopathological result in patients with ESCC, we expected that TNFAIP3 plays a role in cell growth and spread in ESCC. In breast cancer, overexpression of TNFAIP3 induced cell proliferation (19), consistent with a study of hepatocellular carcinoma cells that were reported with increased proliferation of TNFAIP3-overexpressing cells. So it is reasonable when in the present study, the depletion of TNFAIP3 expression in TE-15 cells resulted in a significant reduction in cell proliferation. These results suggest that TNFAIP3 plays a role in the regulation of cell proliferation. Based on a previous study, the mechanism of ABIN is by mimicking NF-κB and combined with TNFAIP3 inhibiting bond formation of TNFAIP3 and NF-κB that cause apoptosis occurrence (12). Different from ABIN mechanism, TNFAIP3 depletion lead to inhibit cell proliferation through attenuation of TNFAIP3 that increased SOCS3 expression, which inhibited STAT3 phosphorylation and suppressed cell proliferation. Because, overexpression of TNFAIP3 regulated lipopolysaccharide/TNF-induced IL-6 inhibition and induced STAT3 phosphorylation. Thus, high and sustained STAT3 phosphorylation induced cell proliferation (30). Moreover, when combined with cisplatin, IC_{50} of cisplatin in TE-15 cells is more cytotoxic compared with combination of cisplatin and TNFAIP3 siRNA treated TE-15 cells (data not shown).

Even though there are no previous reports describing the relationship between TNFAIP3 and the invasive or migratory ability of cancer cells, one study demonstrated microbial invasion of the intestinal inner mucus layer in TNFAIP3 transgenic mice (31). Another report showed that TNFAIP3 influenced the ability of intestinal cell migration in TNFAIP3 knockout mice (32). The present study demonstrated that the invasive and migratory ability of TE-15 cells in the *TNFAIP3* siRNA groups were significantly reduced. Suppression of TNFAIP3 protein inhibits activation of NF-κB, which inhibits the activation of IFNα/STAT1 signaling (32) then resulting in a reduction of cell migration ability.

Clinical report of the TNFAIP3 overexpression is limited. The finding of TNFAIP3 affects in migration and invasion of esophageal cancer cells is a novelty of this study. Investigation to identify a suitable mechanism for cancer therapy is now in progress in order to apply this biomarker into a novel therapy against malignant disease. Furthermore, study that describes TNFAIP3 expression in patients with therapy adjuvant is required in future research.

Our data implied that the expression of TNFAIP3 in ESCC cancer tissues was significantly higher compared with the normal esophageal epithelial tissues, and was significantly correlated with tumor differentiation and found to be an independent prognostic marker for disease survival in ESCC. Our TNFAIP3 knockdown data demonstrated that TNFAIP3 expression may relate, not only to proliferation, but also to migration and invasion in ESCC cells. Taken together, these results suggest that TNFAIP3 could be used as a promising marker for diagnosis and as an effective therapeutic target for ESCC in the future.

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